AD_____

GENETIC AND PHYSIOLOGICAL STUDIES OF BACILLUS ANTHRACIS RELATED TO DEVELOPMENT OF AN IMPROVED VACCINE

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

JULY 1987

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5212

University of Massachusetts
Amherst, Massachusetts 01003

APPROVED FOR PUBLIC RELEASE

DISTRIBUTION UNLIMITED

SECURITY CLASSIFICATION OF THIS PAGE

REP

AD-A187 381

Form Approved
OMB No 0704-0188
Exp. Date: Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION: Unclassified

ARKINGS

2a. SECURITY CLASSIFICATION AUTHORIT

3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION: University of Massachusetts

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION

6c. ADDRESS (City, State, and ZIP Code): Amherst, MA 01003

7b. ADDRESS (City, State, and ZIP Code)

8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research and Development Cmd

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-85-C-5212

8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M161102BS12	AA	106

11. TITLE (Include Security Classification)
(U) Genetic and Physiological Studies of Bacillus anthracis Related to Development of an Improved Vaccine

12. PERSONAL AUTHOR(S): Curtis B. Thorne

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 8/1/86 TO 7/31/87	July 1987	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
05	16	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Bacillus anthracis, B. anthracis plasmids, Bacillus conjugative plasmids, Anthrax protective antigen, Anthrax toxin

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The primary objective of the research is to gain information and to develop genetic systems that will contribute to development of an improved vacccine for anthrax. During the year represented by this report our research concentrated largely on (i) characterization of the conjugative plasmid, pLS20, of B. subtilis and its ability to transfer plasmids among B. subtilis, B. cereus, B. thuringiensis, and B. anthracis; (ii) physical and genetic characterization of the temperate B. thuringiensis phage, TP21, which is active on B. anthracis and whose prophage exists as a plasmid; (iii) further physical and genetic characterization of the B. thuringiensis conjugative plasmids pXO11 and pXO12; (iv) the mechanism of transfer of the B. anthracis plasmids, pXO1 and pXO2, by the conjugative plasmid pXO12; and (v) transformation of B. cereus and B. anthracis with plasmid DNA.

(continued)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION: Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	(301) 663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

An important goal of our research is the development of a system for putting plasmid DNA manipulated *in vitro* back into nontransformable *Bacillus* species such as *B. anthracis*. Our results suggest that plasmid pLS20 of *Bacillus subtilis* (natto), which promotes transfer of the tetracycline resistance plasmid pBC16 from *B. subtilis* to *B. anthracis* and other *Bacillus* species, may be useful for this purpose. In addition to mobilizing pBC16, pLS20 mediates transfer of the *B. subtilis* (natto) plasmid pLS19 and the *Staphylococcus aureus* plasmid pUB110. To facilitate direct selection for pLS20 transfer, plasmid derivatives which carry the erythromycin resistance transposon Tn*917* were generated. It seems likely that the use of pLS20 will facilitate the introduction of plasmid DNA into nontransformable species by use of transformable *B. subtilis* strains as intermediates.

B. thuringiensis subsp. *kurstaki* strain HD1-9 carries a generalized transducing phage, TP-21, whose prophage exists as a 29-Mdal plasmid. Results presented in this report show that cells lysogenized with TP-21 contain the 29-Mdal plasmid and are immune to lysis by TP-21c, a clear-plaque mutant of TP-21. The restriction pattern of DNA isolated from TP-21c phage particles was identical to that of the 29-Mdal plasmid prophage isolated from TP-21 lysogens. The erythromycin resistance transposon Tn*917* was transposed to the TP-21 genome, resulting in a specialized transducing phage carrying the transposon. The Tn*917*-tagged TP-21 was used to determine the host range of the phage. Most strains of *B. anthracis* and *B. thuringiensis* tested were susceptible to the phage. It seems likely that the transposon-tagged TP-21 phage will be a useful tool for transposon mutagenesis in *B. anthracis*.

We have continued studies on the mechanism of conjugative transfer of plasmids mediated by the *B. thuringiensis* fertility plasmids pXO11 and pXO12. These plasmids are able to mediate their transfer and that of other plasmids among *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains, and we are interested in identifying the plasmid genes responsible for conjugative transfer. Results of experiments involving mutant plasmids, restriction analyses, and DNA-DNA hybridizations suggest that the *B. thuringiensis* transposon Tn*4430*, transfer genes, and the parasporal crystal gene are located in the same vicinty on pXO12. DNA-DNA hybridization experiments have shown that the homology exhibited by the four conjugative *B. thuringiensis* plasmids pXO11, pXO12, pXO13, and pXO14 can be attributed in part to the presence of Tn*4430*. The transfer efficiency of pXO12 was determined by using a derivative tagged with Tn*917*; transfer frequencies ranged from 9.2×10^{-3} to 7.1×10^{-2}.

Plasmid pXO12 is able to mediate the transfer of *B. anthracis* plasmids, pXO1 and pXO2, to *B. cereus* and cured *B. anthracis* recipients. In contrast to the relatively high frequency of pBC16 transfer, the number of transcipients which acquired the *B. anthracis* plasmids was significantly lower. In several cases a 4.2-kb DNA sequence originating from pXO12 was present on pXO1 and pXO2 after transfer. The sequence was identified as the *B. thuringiensis* transposon Tn*4430*. Some transcipients harbored cointegrate plasmids of pXO1 and pXO12 which were capable of transferring the PA^+ (protective antigen) phenotype of pXO1 to 50% of the Tc^r transcipients.

We have tried unsuccessfully in the past to transform *B. cereus*, *B. anthracis*, and *B. thuringiensis* by a number of methods that have been reported in the literature. However, a new procedure for transforming *B. thuringiensis* was reported recently (J. Bacteriol. 169:1147-1152 [1987]), and we have had some success in adapting the method to *B. cereus*. We are optimistic that the method will be applicable to *B. anthracis*.

AD_____

GENETIC AND PHYSIOLOGICAL STUDIES OF BACILLUS ANTHRACIS RELATED TO DEVELOPMENT OF AN IMPROVED VACCINE

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

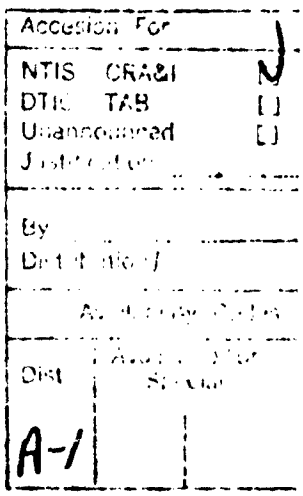
JULY 1987

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5212

University of Massachusetts
Amherst, Massachusetts 01003



APPROVED FOR PUBLIC RELEASE

DISTRIBUTION UNLIMITED

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SUMMARY

This is a progress report (second annual report) of research being carried out on Bacillus anthracis under contract DAMD17-85-C-5212. Research on the contract which began August 1, 1985 is a continuation of research previously carried out under contract DAMD17-80-C-0099. During the year represented by this annual report our research concentrated largely on (i) characterization of the conjugative plasmid, pLS20, of B. subtilis and its ability to transfer plasmids among B. subtilis, B. cereus, B. thuringiensis, and B. anthracis; (ii) physical and genetic characterization of the temperate B. thuringiensis phage, TP21, which is active on B. anthracis and whose prophage exists as a plasmid; (iii) further physical and genetic characterization of the B. thuringiensis conjugative plasmids pXO11 and pXO12; (iv) the mechanism of transfer of the B. anthracis plasmids, pXO1 and pXO2, by the conjugative plasmid pXO12; and (v) transformation of B. cereus and B. anthracis with plasmid DNA.

The 55-kb plasmid, pLS20, of Bacillus subtilis (natto) 3335 promotes transfer of the tetracycline resistance plasmid pBC16 from B. subtilis (natto) to B. anthracis, B. cereus, B. licheniformis, B. megaterium, B. pumilus, B. subtilis, and B. thuringiensis. Frequency of pBC16 transfer ranged from 2.3 x 10^{-6} to 2.8 x 10^{-3}. Evidence for a plasmid-encoded conjugation-like mechanism of genetic exchange includes: (1) pLS20^{+} strains, but not pLS20^{-} strains, functioned as donors of pBC16, (2) Plasmid transfer was insensitive to the presence of DNase, and (3) Cell-free filtrates of donor cultures did not convert recipient cells to Tc^{r}. Cotransfer of pLS20 and pBC16 in intraspecies matings and in matings with a restriction-deficient B. subtilis strain indicates that pLS20 is self-transmissible. In addition to mobilizing pBC16, pLS20 mediated transfer of the B. subtilis (natto) plasmid pLS19 and the Staphylococcus aureus plasmid pUB110. The fertility plasmid does not carry a selectable marker. To facilitate direct selection for pLS20 transfer, plasmid derivatives which carry the erythromycin resistance transposon Tn917 were generated. Development of this method of genetic exchange will facilitate the introduction of plasmid DNA into nontransformable species by use of transformable fertile B. subtilis or B. subtilis (natto) strains as intermediates.

Contrary to reports in the literature that B. thuringiensis subsp.

kurstaki HD1-9 carries a 29-Mdal plasmid that will mediate conjugative transfer of chromosomal genes to B. cereus, our results show that transfer of chromosomal genes during mating with that strain as donor occurs by means of transduction. The strain HD1-9 carries two or more generalized transducing phages. The prophage of on f these exists as a 29-Mdal plasmid. We have named the phage TP-21. Results presented here show that phage TP-21 isolated from B. thuringiensis subsp. kurstaki HD1-9 will lysogenize B. cereus and the resulting lysogens contain the 29-Mdal plasmid and are immune to lysis by TP-21c, a clear plaque mutant of TP-21. The restriction patterns of DNA isolated from TP-21c phage and the 29-Mdal plasmid prophage isolated from B. cereus 569 TP-21 lysogens were identical. The erythromycin resistance transposon Tn917 was transposed to the TP-21 genome, resulting in a specialized transducing phage carrying the transposon. TP-21::Tn917 was used to determine the host range of TP21. Most strains of B. anthracis and B. thuringiensis tested were susceptible to the phage. It seems likely that the transposon-tagged TP-21 phage will be a useful tool for transposon mutagenesis in B. anthracis and other susceptible organisms.

We reported previously on the identification of two self-transmissible plasmids, pXO11 (56 Mdal) and pXO12 (75 Mdal), indigenous to B. thuringiensis 4042A subsp. thuringiensis. These plasmids are able to mediate conjugative transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis strains, and we are interested in identifying the plasmid genes responsible for conjugative transfer. Restriction analysis of two crystalliferous (Cry^+), transfer deficient (Tra^-) deletion derivatives of pXO12 has shown that DNA sequences involved in conjugal transfer functions are located on a 48.5-kb AvaI fragment. DNA-DNA hybridization experiments have shown that a copy of the B. thuringiensis transposon, Tn4430, is located on a 5.7-kb BglII fragment contained within the 48.5-kb AvaI fragment of pXO12. Analysis of two Cry^- Tra^+ derivatives of pXO12 has indicated that a 16.0-kb AvaI fragment encodes information required for the synthesis of parasporal crystals, and the 16.0-kb and the 48.5-kb AvaI fragment are adjacent on pXO12. Based on these results, it appears that Tn4430, the transfer genes, and the parasporal crystal gene are located in the same vicinty on pXO12. DNA-DNA hybridization experiments have shown that the homology exhibited by the four conjugative B. thuringiensis plasmids pXO11, pXO12, pXO13 and pXO14 can be attributed in part to the presence of Tn4430. The transfer efficiency of pXO12 has been

determined by using marked derivatives of pXO12 which carry the Em^r transposon Tn917; transfer frequencies ranged from 9.2×10^{-5} to 7.1×10^{-2}.

Plasmid pXO12, is able to mediate the transfer of B. anthracis plasmids, pXO1 and pXO2, into B. cereus and cured B. anthracis recipients. In contrast to the relatively high frequency of pBC16 transfer, the number of transcipients which acquired the B. anthracis plasmids was significantly lower. In several cases a 4.2-kb DNA sequence originating from pXO12 was found to have transposed onto pXO1 and pXO2 after transfer. This 4.2-kb sequence was determined to be identical to the recently described B. thuringiensis transposon Tn4430. Transcipients were also obtained which harbored cointegrate plasmids of pXO1 and pXO12 which were capable of transferring the PA^+ (protective antigen) phenotype of pXO1 to 50% of the total Tc^r transcipients.

In recent years a number of reports have appeared in the literature on methods for transforming B. thuringiensis. We have tried unsuccessfully to transform B. cereus, B. anthracis, and B. thuringiensis by most, if not all, of the methods that have been reported. This has been the experience with most laboratories that have tried to use the tranformation methods that have been reported. Heierson, et al. (J. Bacteriol. 169:1147-1152 [1987]) recently reported a new method for transforming B. thuringiensis, and we have had some success in adapting their procedure to B. cereus. We are optimistic that the method will be applicable to B. anthracis.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

ANNUAL PROGRESS REPORT

This is the second annual report submitted under contract DAMD17-85-C-5212. Research on the contract which began August 1, 1985 is a continuation of research previously carried out under contract DAMD17-80-C-0099.

During the year represented by this annual report our research concentrated largely on (i) characterization of the conjugative plasmid, pLS20, of B. subtilis and its ability to transfer plasmids among B. subtilis, B. cereus, B. thuringiensis, and B. anthracis; (ii) physical and genetic characterization of the temperate B. thuringiensis phage, TP21, which is active on B. anthracis and whose prophage exists as a plasmid; (iii) further physical and genetic characterization of the B. thuringiensis conjugative plasmids pXO11 and pXO12; (iv) the mechanism of transfer of the B. anthracis plasmids, pXO1 and pXO2, by the conjugative plasmid pXO12; and (v) transformation of B. cereus and B. anthracis with plasmid DNA.

In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. Table 1 lists the strains and mutants referred to in this report.

Media. For convenience to the reader, compositions of the various culture media referred to in this report are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

$NBYCO_3$ agar: NBY agar with 7 g of $NaHCO_3$.

Phage assay (PA) broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2.2H_2O$, 0.15 g. The pH was

adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

$PACO_3$ agar: PA agar with 7 g of $NaHCO_3$.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

LG broth: L broth with 1 g of glucose.

BHI broth: Brain heart infusion broth (Difco), 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Peptone diluent: Peptone (Difco), 10 g. Used for diluting phage and bacterial cells.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal 3: To Minimal 1 was added 10 mg of thiamine hydrocloride and 200 mg of glycine.

Minimal 4NH: To Minimal 3 was added 40 mg of L-methionine and L-histidine, and 10 mg of nicotinamide.

SG medium: This medium for polyglutamate production by B. subtilis (natto) was described by Hara et al. (12). It contained L-glutamic acid (monosodium salt), 15 g; KH_2PO_4, 2.7 g; $Na_2HPO_4.12H_2O$, 4.2 g; NaCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; sucrose, 50 g; and biotin, 1 mg. The pH was adjusted to 6.4.

DM3 medium: This medium for regenerating protoplasts in transformation experiments was prepared as described by Chang and Cohen (7).

CA-agarose medium: CA-agarose medium for the detection of colonies producing protective antigen was prepared as follows: 0.75 g of agarose was added to 100 ml of CA broth (prepared as described by

Thorne and Belton [37]) and the mixture was steamed until the agarose was dissolved. When the medium cooled to about 50°C, 1 ml of 20% glucose, 8 ml of 9% $NaHCO_3$, 6 ml of goat antiserum to B. anthracis, and 10 ml of horse serum were added. The medium was dispensed in petri plates (13 ml per plate) and the plates were left with their lids ajar while the agarose solidified. The plates were usable after 1 hr.

Antisera. B. anthracis antiserum was kindly supplied by personnel of USAMRIID.

Test for auxotrophic markers. To test for auxotrophic markers of transcipients, the minimal media Min I and Min IC, were supplemented as appropriate with the required amino acids, purines, or pyrimidines at a concentration of 40 µg/ml. Biotin (0.1 µg per ml) was added to minimal media for growth of B. subtilis (natto).

Propagation and assay of bacteriophage CP-51 and CP-54. The methods described previously (28, 31, 33) were followed. The indicator for routine assay of these phages was B. cereus 569.

Propagation and assay of phage TP-21 and TP-21c lysates. Cell-free lysates of TP-21 were prepared by growing cultures of lysogens from a loop of cells or spores in 25 ml BHI broth with 0.1% glycerol. Following 16 h of incubation at 30°C the cultures were centrifuged to remove the cellular debris and filtered through a 0.45-µm HA Millipore filter. Lysates of TP-21c were prepared by lytic infection of B. cereus 569 in broth cultures. All lysates were confirmed to be free of bacterial contamination by plating samples on L agar.

TP-21 and TP-21c were assayed by the soft agar overlay technique on NBY or PA agar, with spores of B. cereus 569 as the indicator. Phage was diluted in 1% peptone and 0.1 ml was mixed with 0.1 ml of indicator in 2 ml of soft agar. Sometimes 0.1 % glycerol (w/v) was added to the top agar to improve the ability to detect plaques of phage TP-21. Plates were incubated at 30°C and plaques were counted after 20 h.

Isolation of phage TP-21 DNA. The phage from a lysate containing 10^{10} or more PFU per ml was collected by centrifugation for 2 h at 25,000 rpm. The phage pellet from each 65 ml of culture was then suspended in 1 ml of SSC buffer and pooled with the other phage suspensions. An equal volume of freshly distilled phenol saturated with SSC was added and the tube was gently rolled for 10 min. The mixture was centrifuged at 10,000 rpm for 30 min. The phenol layer

was removed and the aqueous layer was entrifuged at 10,000 rpm for 20 min to pellet the protein. The aqueous phase was poured into a sterile tube and the deproteinization procedure was repeated. The DNA was precipitated by first adding one half volume of 7.5 M ammonium acetate and mixing well. Two volumes of 95% ethanol were added and the tube contents were mixed well by inverting the tube 20 times. The tube was held on ice for 10 min and then the DNA pellet was collected by centrifuging at 0°C for 1 h at 10,000 rpm. The ethanol was decanted and the DNA pellet was allowed to drain over paper towels for 5 to 10 min before resuspending in 5 ml of TES (50 mM Tris-HCl, 50 mM NaCl, 3 mM EDTA [pH 8.0]).

Test for capsule production. The ability of B. anthracis and B. cereus to produce capsules was determined by growing cells on PA agar incubated in air, or on $PACO_3$ agar incubated in 20% CO_2. Plates were incubated at 37°C for 24 to 48 h.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (16). Cells were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10 µg/ml). With some strains better results were obtained when 0.5% (w/v) glycerol was included in the BHI broth to prevent sporulation. Best results with strains of B. anthracis were obtained when the BHI broth was supplemented with 10% (v/v) horse serum. The inoculum for each flask was a loop of growth from an L agar plate which had been streaked with a loop of spores and incubated at 37°C for 16 to 24 hours. Cultures were incubated at 37°C on a rotary shaker (100 to 160 rpm) for 13 to 16 hours. Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and resuspended in 1 ml of E buffer (0.04 M Tris-OH (Sigma), 0.002 M EDTA (tetrasodium salt, Sigma), 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer prepared by adding 3 gm of sodium dodecyl sulfate and 5.0 ml of 3.0 N NaOH to 100 ml of 15% (w/v) sucrose in 0.05 M Tris-OH. The tubes were rapidly inverted 20 times to mix the cells and buffer and they were then held in a 60°C water bath for 30 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C and the aqueous phase was removed for electrophoresis.

The procedure as described above was used for B. anthracis, B. cereus, and B. thuringiensis. It was modified slightly for B. subtilis and B. licheniformis. Cultures were grown for 16 hours in BHI broth supplemented with 0.1% glycerol, or in LG broth for B. subtilis (natto). After cell pellets were suspended in 2 ml of E buffer, lysozyme was added to give a final concentration of 2 mg per ml, and suspensions were incubated at 37°C for 45 to 60 minutes. Two ml of lysis buffer as described above were added and tubes were inverted 20 times. After the suspensions were incubated 30 minutes at 60°C they were cooled on ice and 0.5 ml of 2M Tris-OH was added. The tubes were inverted 20 times, and following the addition of 6 ml of cold phenol-chloroform mixture to each tube, they were inverted 20 times again. Finally the tubes were centrifuged at 10,000 rpm for 10 minutes and the aqueous layer was withdrawn.

For electrophoresis of plasmid DNA, extracts (40 µl) were mixed with 10 µl of tracking dye (0.25% bromphenol blue, 15% ficoll) and samples (40 µl) were applied to horizontal 0.7% agarose (Sigma, Type II medium EEO) gels prepared and run in Tris-borate buffer (0.089 M Tris-OH, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3). Electrophoresis was carried out at 70 V for 90 to 120 min at room temperature. Gels were stained with ethidium bromide (1 µg/ml) in Tris-borate buffer).

Method for isolating plasmid DNA suitable for restriction analysis. The above procedure for extracting plasmid DNA has been modified in such a way that very little or no chromosomal DNA is present in the preparations. At least there is not enough chromosomal DNA present to interfere with restriction analysis of plasmids. The modified procedure is the same as the procedure described above through the lysis step at 60°C. At that point cell debris and unlysed cells were removed by centrifugation at 10,000 rpm for 15 minutes at 5°C. The supernatant fluid was decanted and placed in an ice bath. Ice-cold 2 M Tris (0.5 ml, pH 7.0) was added to neutralize the lysate and mixing was accomplished by inverting the tubes 20 times. The lysate was extracted with 6 ml of cold phenol-chloroform by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 minutes at 5°C, and the aqueous phase was removed. One-half volume of 7.5 M ammonium acetate was added, and the plasmid DNA was precipitated by adding twice the final volume of ice cold 95% ethanol. The tubes were held on ice for 15 minutes and then centrifuged at 10,000 rpm for 30 to 60 minutes at 5°C. The ethanol was decanted, 5 ml of cold 70% ethanol was added to each tube which was then mixed

gently on a vortex mixer, and the DNA was collected by centrifugation at 10,000 rpm for 15 minutes at 5°C. After the ethanol was decanted, the tubes were inverted over paper towels for 10 minutes and then placed in a vacuum desiccator for at least 2 hours to dry the DNA thoroughly. The DNA was dissolved in 0.1 to 0.5 ml of TES (0.05 M Tris-OH, 0.05 M NaCl, 0.005 M EDTA, pH 8.0) containing 50 µg of RNase per ml.

If larger preparations of plasmid DNA are desired, the above procedure can be scaled up successfully.

Plasmid DNA for nick translation was purified further by cesium chloride density gradient centrifugation.

Restriction endonuclease digestions. Restriction endonuclease digestions were carried out under conditions recommended by the supplier of the enzymes. Usually 10 to 20 µl of DNA (1.0 to 1.5 µg) in TES (pH 8.0) was added to 5 to 10 units of enzyme in a 1.5 ml Eppendorf tube. Appropriate amounts of distilled water and 10X buffer were added to give a total volume of 100 µl. Reaction mixtures were incubated in a 37°C water bath for 2 to 15 h. Digests were heated at 65°C for 10 minutes to stop reactions and then resolved on agarose gels.

Transduction of pXO2. Bacteriophage CP-51ts45 was propagated on B. anthracis and assayed on B. cereus 569. Recipient cells for transduction were grown in 250-ml flasks containing 25 ml of L broth (for B. cereus) or BHI broth with 0.5% glycerol (for B. anthracis) and incubated at 37°C on a rotary shaker at 250 rpm. Cells from a 10% (vol/vol) transfer of a 16-h culture were grown for 5 h. Cells (0.1 ml containing approximately 10^8 CFU) and phage (0.1 ml containing approximately 5×10^9 PFU) were spread together on $NBYCO_3$ or $PACO_3$ agar. Plates were incubated at 37°C in 20% CO_2. After 3 h, 0.1 ml of phage CP-54 (3×10^9 PFU) was spread on the transduction plates to lyse noncapulated cells and to allow the selection of capsulated transductants. Incubation in CO_2 was continued for 36 to 48 h.

Protoplast transformation. B. subtilis and B. subtilis (natto) were transformed by the method of Chang and Cohen (7).

Procedures used in mating experiments:

(1) Matings in broth: Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared

by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml. If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10 µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

(2) Matings on membranes: Donor and recipient cells were grown in 250-ml flasks containing 25 ml of BHI broth and incubated at 30°C on a reciprocal shaker, 80 excursions per min. To prevent cell clumping, B. licheniformis recipient cultures were grown at 37°C, 250 rpm. Transfers (5%, v/v) from 14- to 16-h cultures were grown for 5 h. One ml of donor cells and 1 ml of recipient cells were mixed and 0.1-0.2-ml samples were spread onto Millipore DA or HA membranes (Millipore Corp., Bedford, MA) which were placed on nonselective medium for 5 hr. BHI agar was usually used if the recipients were B. anthracis, B. cereus, or B. thuringiensis. PA agar was usually used for B. subtilis, and LG agar was used when the matings involved B. subtilis (natto). To determine the number of donor and recipient cells per membrane, the mixture was diluted in peptone and plated on the appropriate selective media. Control mixtures contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Plates were

incubated at 30°C for 5 h to allow mating and phenotypic expression. Membranes were subsequently transferred to agar plates containing tetracycline (for pBC16 transfer) or neomycin (for pUB110 transfer) and either rifampicin or streptomycin to select for recipients which had acquired the antibiotic resistance plasmid from the donor. To select for transfer of Tn917-containing plasmids, membranes were transferred to agar containing erythromycin and lincomycin, and either rifampicin or streptomycin. Colonies were scored after 1 to 2 days of incubation and transcipients were purified on the selective medium. The use of auxotrophically marked strains facilitated unambiguous identification of transcipients. Frequency is expressed as the number of transcipients per donor.

Screening colonies for protective antigen production. Colonies were picked to plates of CA-agarose medium and incubated at 37°C in 20% CO_2 for about 16 h. A zone of precipitate formed around colonies that produced protective antigen.

Southern blotting and hybridization. DNA was purified by isopycnic centrifugation in cesium chloride gradients. The DNA was radiolabelled by nick translation (27) using [α-^{32}P]-dGTP purchased from Amersham, Arlington Heights, IL, and a kit obtained from Bethesda Research Laboratories. Plasmid DNA restriction fragments separated on a 0.6% agarose gel were transferred to GeneScreen Plus nylon membranes (New England Nuclear Co.) by the Southern blotting technique (29). The DNA-DNA hybridization protocol was that recommended by the supplier.

Screening colonies for fertility. A replica plate mating technique was employed to screen large numbers of transcipients for fertility. Colonies of transcipients to be tested were picked to BHI agar to form master plates. These were incubated 16 to 18 h at 30°C and the colonies were replica plated to BHI agar plates that had been spread with 0.1 ml of spores (approx 1 x 10^8 CFU) of a recipient strain. The Str^r strains, B. anthracis UM44-1C9 and B. cereus UM20-1, and the Rif^r strain, B. anthracis UM23C1-2, were used as recipients. The plates were incubated 16 to 18 h at 30°C and the mixed growth was then replica plated to agar plates containing tetracycline and the appropriate antibiotic to select for the recipient strain. Incubation at 30°C was continued. After 16 to 20 h, patches of transcipient growth were present in areas corresponding to particular colonies of transcipients on the master plate which were fertile.

RESULTS AND DISCUSSION

I. Characterization of the conjugative plasmid, pLS20, of B. subtilis

Previous reports from our laboratory have described systems for plasmid transfer in and among strains of Bacillus anthracis, B. cereus, and B. thuringiensis. CP-51-mediated transduction and matings employing self-transmissible plasmids from B. thuringiensis facilitate plasmid transfer among these three Bacillus species (4, 28, 35). However, these genetic exchange systems are not suitable for plasmids constructed in vitro or those carried by strains outside the host range of CP-51 and the B. thuringiensis fertility plasmids. Therefore, a plasmid-encoded mating system has been developed for transfer of plasmid DNA from the readily transformable Bacillus species, B. subtilis and B. subtilis (natto), to B. anthracis and other Bacillus species for which efficient transformation systems have not been developed.

B. subtilis (natto) strains contain a variety of endogenous plasmids. In 10 of 15 strains screened by Tanaka and Koshikawa (30), one or more plasmids ranging from 5.4 to 69 kb were found. Strain 3335 harbors a 54.65-kb plasmid, pLS20, and a 5.4-kb plasmid, pLS19. pLS19 has been reported to be associated with polyglutamate production in this species (12-14). The function of pLS20 in strain 3335 has not been determined previously.

pLS20 or other plasmids of similar size are present together with 5.4 kb or 6.0 kb plasmids in B. subtilis (natto) strains. To account for the array of plasmid profiles in various B. subtilis (natto) isolates, Tanaka and Koshikawa (30) have suggested that plasmids may be transferred between strains by some unknown mechanism. B. subtilis (natto) strains are isolated from a vegetable cheese prepared by fermentation of boiled soybeans. Thus, during fermentation cells have the opportunity for contact. These considerations, plus reports by Carlton and Gonzalez (6) and findings in our own laboratory (4, 26) demonstrating the presence of large self-transmissible plasmids in B. thuringiensis, prompted investigation of a possible role of pLS20 in mating.

Plasmid content of B. subtilis (natto) strains. B. subtilis (natto) strain 3335 harbors the 5.4-kb plasmid pLS19 and the 54.6-kb plasmid pLS20. Fig. 1A shows agarose gel electrophoresis of plasmid lysates of strain 3335 and some derivatives generated in our laboratory. The spontaneously cured isolate, UM4, carries only pLS20. The 4.2-kb tetracycline resistance plasmid pBC16 (5) was

introduced into UM4 by transformation to yield the Tc^r strain UM8. B. subtilis (natto) UM24 is cured of all plasmids.

Transfer of pBC16. B. subtilis (natto) strains were tested for the ability to transfer plasmid pBC16 in intraspecific and interspecific matings. Fig. 1B shows agarose gel electrophoresis of plasmid DNA from donor, recipient, and transcipient strains from matings with cured B. subtilis (natto) and B. anthracis recipients. When B. subtilis (natto) UM8(pLS20, pBC16) was mated with B. subtilis (natto) UM24, 1.7×10^4 Tc^r transcipients were obtained. All of 16 Tc^r transcipients examined acquired pBC16. Seven of these transcipients also acquired pLS20 from the donor. Tc^r $pBC16^+$ trancipients were also obtained in a mating with B. anthracis UM23C1-2 as the recipient. However, cotransfer of pLS20 to B. anthracis was not observed in these matings. Of 388 B. anthracis transcipients examined by plasmid extractions and/or replica plate matings, none was $pLS20^+$. pLS20 has recently been transferred to B. anthracis in an experiment in which recipient cells were heated to 51°C prior to mating.

Tc^r transcipients were obtained only when strains harboring pLS20 were used as donors. All Tc^r transcipients retained the auxotrophic marker of the recipient strain. Transfer of tetracycline resistance was not observed from $pLS20^-$ $pBC16^+$ strains, such as B. subtilis (natto) UM28.

Mechanism of plasmid transfer. To investigate the possibility of transformation of pBC16 into recipient cells, the sensitivity of plasmid transfer to DNase was tested. B. subtilis (natto) UM8(pLS20, pBC16) and B. anthracis UM44-1C9 cultures were incubated in the presence of DNase. Cultures were then mixed and spread onto membranes with additional DNase. The number of Tc^r transcipients obtained (4.4×10^3) after incubation with DNase and $MgSO_4$ did not differ significantly from the number of transcipients obtained (2.7×10^3) after incubation with $MgSO_4$ alone.

To determine whether plasmid transfer occurred by transduction, the ability of cell-free filtrates of B. subtilis (natto) UM8 cultures to convert B. anthracis UM44-1C9 to tetracycline resistance was investigated. Filtrates were prepared from mitomycin C- and UV-induced donor cultures and from an untreated donor culture. No Tc^r transcipients were detected when the filtrates were mixed with recipient cells and spread onto membranes.

Participation of a wide variety of Bacillus species in pLS20-mediated matings. Table 2 shows the results of matings between B. subtilis (natto) UM8(pLS20, pBC16) and several Bacillus species and strains. pBC16 was

transferred to all species tested. Transfer of pBC16 was confirmed by plasmid extraction of Tc^r transcipients. All Tc^r transcipients tested acquired pBC16 and retained the genetic markers of the respective recipient strain. Colonies on membranes represented independent transcipients.

Matings of B. subtilis (natto) with B. anthracis, B. cereus, and B. thuringiensis, resulted in comparable numbers of transcipients per ml (1.3×10^3 to 3.4×10^3). The greatest numbers of Tc^r transcipients/ml were obtained in matings with B. licheniformis (1.4×10^4), B. pumilus (1.4×10^4), B. subtilis 168 (9.5×10^4), and in intraspecies matings in which pBC16 was transferred to a cured isolate of B. subtilis (natto) (1.7×10^4). B. megaterium was a relatively inefficient recipient; 3.5×10^1 transcipients/ml were detected and the frequency of transfer was 2.3×10^{-6}. The low number of B. subtilis W23 transcipients/ml (3.8×10^2) and the high value obtained for the frequency of transfer to this strain were attributed to killing of donor cells by the recipient, as indicated by cell counts (data not shown).

Cotransfer of pLS20 and pBC16. Transfer of pLS20 from B. subtilis (natto) was observed only in intraspecies matings and in matings with the restriction-deficient B. subtilis strain PSL1 UM1. As indicated in Table 3, when B. subtilis (natto) UM8(pLS20, pBC16) was mated with the cured B. subtilis (natto) strain UM24, 43.8% of the Tc^r transcipients tested acquired pLS20 in addition to pBC16. When the same donor was mated with B. subtilis PSL1, 28.6% of the Tc^r transcipients tested obtained pLS20. Cotransfer of pLS20 with pBC16 from B. subtilis (natto) to B. subtilis 168 UM21 and to B. subtilis IG-20 was not detected. Similarly, transfer of pLS20 from B. subtilis (natto) to other Bacillus species was not observed (data not shown).

The restriction-deficient B. subtilis strain, IG-20, proved to be a poor recipient for transfer of pLS20 from B. subtilis and from B. subtilis (natto). pLS20 was transferred at a low frequency (1.5%) from B. subtilis PSL1 UM3 to B. subtilis IG-20. However, pLS20 was transferred from a resulting B. subtilis IG-20 transcipient, UM3, to B. subtilis 168 at a high frequency. In this mating, 30.5% of the Tc^r transcipients tested carried the fertility plasmid.

Cotransfer of pLS19 and pBC16. In addition to mediating transfer of pBC16, pLS20 promoted transfer of the 5.4-kb plasmid, pLS19, of B. subtilis (natto) 3335. Since it was not possible to select for pLS19 transfer, we looked for cotransfer of the plasmid with pBC16. B. subtilis (natto) UM31 harboring pLS20, pLS19, and pBC16 was mated with a cured strain of B. subtilis (natto), B.

anthracis, and various B. subtilis strains. Tc^r transcipients were examined for plasmid content by plasmid extraction. Fig. 2 shows the plasmid profiles of the donor strain, the B. subtilis (natto) recipient, UM24, and three representative transcipients. In this intraspecies mating, 26.7% of the Tc^r B. subtilis (natto) transcipients tested acquired pLS19 in addition to pBC16. In matings with the restriction-deficient B. subtilis strains, IG-20 UM2 and PSL1 UM1, 46.7% and 44.4%, respectively, of the $pBC16^+$ transcipients were $pLS19^+$. Frequency of cotransfer of pLS19 with pBC16 to B. anthracis UM44-1C9 was 12.5%, and 6.7% of B. subtilis 168 UM21 Tc^r transcipients were $pLS19^+$. As expected from results presented above, cotransfer of pLS20 from the B. subtilis (natto) donor to B. subtilis (natto) and B. subtilis PSL1 transcipients was also observed.

5.4-kb plasmids, such as pLS19, in various B. subtilis (natto) strains have been reported to be associated with polyglutamate (PGA) production (12-14). Tc^r B. subtilis (natto) and B. subtilis transcipients were tested for the Pga^+ phenotype by growing the isolates on SG medium. We did not find any correlation between the presence of pLS19 and PGA production. None of the $pLS19^+$ transcipients acquired the ability to produce PGA (data not shown).

$pLS20^+$ B. subtilis transcipients as donors of pBC16 and pUB110. B. subtilis PSL1 UM12, which acquired pLS20 and pBC16 in a mating with B. subtilis (natto) UM8, was tested for its ability to transfer pBC16 to B. anthracis, B. subtilis, and B. subtilis (natto). Results shown in Table 4 indicate that the presence of pLS20 in B. subtilis PSL1 rendered this strain transfer-proficient. The numbers of Tc^r transcipients per ml obtained in these matings are comparable to those obtained when a B. subtilis (natto) donor was mated with these species (see Table 2).

B. subtilis PSL1 UM12 was cured of pBC16 and transformed with the neomycin resistance plasmid pUB110 (10). The resulting strain, PSL1 UM11, was tested as a donor of this plasmid. Results presented in Table 4 show that this donor transferred pUB110 at frequencies similar to those for pBC16 transfer. Plasmid extractions of 8 Nm^r transcipients from each mating confirmed the acquisition of pUB110.

Isolation of pLS20::Tn917 derivatives. A B. subtilis transcipient carrying pLS20 and pBC16 was transformed with the transposition selection vector, pTV1. This 12.4-kb plasmid contains the Streptococcus faecalis transposon Tn917 which carries an erythromycin-inducible gene for resistance to macrolides,

lincosamides, and streptogramin B antibiotics (MLS resistance). Plasmid pTV1 also carries a chloramphenicol resistance determinant and is temperature sensitive for replication (42). The transformed strain, B. subtilis PSL1 UM3 (pLS20, pBC16, pTV1), was inoculated into 25 ml of L broth containing chloramphenicol, erythromycin, and lincomycin and incubated at 30°C, with slow shaking (80 rpm). After 24 h, a 5% transfer was made to the same medium and incubation was continued at 30°C for 5 h. A 1% transfer was made into LG broth containing only erythromycin and lincomycin and the culture was incubated at 48°C with slow shaking (100 rpm). After 24 h, the 1% transfer was repeated and the culture was incubated overnight at 48C.

Cells from the induced culture described above were mated with B. subtilis IG-20 UM1. Mating mixtures were spread on DA membranes placed on LG agar. Plates were incubated at 30°C for 3 h to allow transfer of plasmid DNA. Membranes were subsequently transferred to LG agar containing erythromycin (0.1 µg/ml) to induce MLS resistance. After 2 h of incubation, membranes were placed on LG agar containing erythromycin (1 µg/ml), lincomycin (10 µg/ml) and rifampicin (10 µg/ml). Colonies were scored after 3 days and transcipients were streaked for single colony isolation. At the time of mating, the donor culture contained 1.5×10^7 MLS^r cfu/ml and 1.1×10^3 Cm^r cfu/ml, indicating a 99.99% loss of pTV1. Four MLS^r transcipients were obtained: B. subtilis IG-20 UM4, IG-20 UM5, IG-20 UM6, and IG-20 UM7. All of these were Cm^s, indicating that pTV1 had not been transferred.

Fig. 3 shows the plasmid profiles of the PSL1 donor, the IG-20 recipient, and one of the resulting transcipients, B. subtilis IG-20 UM6. Plasmid extractions revealed that each of the MLS^r transcipients carried a large molecular weight plasmid which comigrated with pLS20. These plasmids have been designated pXO501, pXO502, pXO503, and pXO504. Two of the isolates, IG-20 UM6 and IG-20 UM7, had also acquired pBC16 and were Tc^r.

Transfer of pLS20::Tn917 derivatives. The B. subtilis IG-20 MLS^r transcipients, UM4 (pXO501), UM5 (pXO502), UM6 (pXO503, pBC16), and UM7 (pXO504, pBC16), were tested for the ability to transfer MLS resistance by mating. The data presented in Table 5 demonstrate that each of the isolates was fertile. In matings with B. subtilis 168 UM21, the number of MLS^r transcipients/ml ranged from 2.0×10^2 to 1.0×10^3. Fig. 3 shows the plasmid content of an MLS^r transcipient, B. subtilis 168 UM42, obtained from a mating with B. subtilis IG-20 UM6. All MLS^r transcipients tested had acquired a large molecular weight

plasmid, and in matings employing the pBC16$^+$ donors, cotransfer of pBC16 was also observed.

The pBC16$^+$ MLS^r donors, B. subtilis IG-20 UM6 and IG-20 UM7, were tested in similar matings in which selection was for transfer of pBC16. The results shown in Table 5 indicate that these strains transferred pBC16 to B. subtilis 168 UM21 at frequencies of 1.1×10^{-4} to 2.4×10^{-3}, compared to a frequency of 4.4×10^{-3} when a donor carrying the unmarked plasmid was tested. Cotransfer of the large molecular weight plasmids, pXO503 and pXO504, was observed, and all of these transcipients were MLS^r.

These results indicate that the erm gene of Tn917 can serve as a selectable marker on pLS20, and we can now select directly for transfer of the fertility plasmid. One of the Tn917-carrying pLS20 derivatives, pXO503, was chosen for further study. This plasmid was transferred from B. subtilis 168 UM42 to B. anthracis UM23C1-2 by selecting for MLS^r transcipients. The frequency of transfer of pXO503 to B. anthracis was 2.7×10^{-6}; only 4 MLS^r transcipients were obtained. However, all of the transcipients acquired the fertility plasmid and retained the genetic marker of the recipient.

Restriction analysis of pLS20 and pXO503 and hybridization with pTV1. Results of EcoRI-digestion of pLS20 DNA from B. subtilis PSL1 UM4 and pXO503 DNA from B. subtilis 168 UM42 are shown in Figure 4A. The sizes of restriction fragments for each plasmid are listed in Table 6. Digestion of pLS20 with EcoRI resulted in 17 fragments ranging in size from 1.3 to 7.25 kb. The sum of the fragment sizes indicated a plasmid size of 54.65 kb. EcoRI-digestion of pXO503 DNA demonstrated that pXO503 contained 5.15 kb of DNA not present in pLS20. As indicated in Fig. 4A and Table 6, the pXO503 digest did not contain a 4.1-kb fragment (no. 5) found in the pLS20 digest. However, a larger fragment (no. 1A, 9.25 kb) appeared in the digest of pXO503. The difference in size of the two fragments corresponds to the size of Tn917.

The EcoR1-digested DNA was transferred after electrophoresis to a nylon membrane and hybridized with ^{32}P-labelled pTV1 DNA. The autoradiograph presented in Fig. 4B, shows that pTV1 DNA hybridized with the altered band in the pXO503 digest, indicating that Tn917 had inserted into the 4.1-kb fragment from pLS20.

Discussion. The results presented here demonstrate that the 55-kb plasmid, pLS20, of B. subtilis (natto) 3335 promotes transfer of the tetracycline resistance plasmid pBC16 to the Bacillus species anthracis, cereus,

licheniformis, megaterium, pumilus, subtilis, and thuringiensis. Evidence indicating that pLS20 was responsible for plasmid transfer includes: (1) pLS20$^+$ strains, but not pLS20$^-$ strains, functioned as donors of pBC16; (2) Incubation of donor and recipient cultures in the presence of DNase, followed by mating in the presence of DNase did not affect plasmid transfer; and (3) Cell-free filtrates of donor cultures did not convert recipient cells to Tc^r.

Plasmid pLS20 does not carry a known selectable marker. Therefore, it was not possible to select for pLS20$^+$ transcipients. Cotransfer of pLS20 with pBC16 from donors carrying only pLS20 and pBC16 demonstrates that the plasmid is self-transmissible. Acquisition of pLS20 by B. subtilis transcipients rendered this species transfer-proficient, thus providing further proof that pLS20 is conjugative. Cotransfer of the fertility plasmid with pBC16 from B. subtilis (natto) was observed only in intraspecies matings and in matings with a restriction-deficient B. subtilis strain. These observations and recent experiments in which pLS20 was transferred to B. anthracis cells which were heated prior to mating suggest that pLS20 may be mobilized into various recipient strains, but is subject to restriction.

To facilitate direct selection for pLS20 transfer, pLS20 derivatives which carry the erythromycin resistance transposon Tn917 as a selectable marker were generated. The transposition-selection vector pTV1, constructed by Youngman (42), was employed to introduce Tn917 into a fertile B. subtilis strain. Four isolates carrying a transposon-tagged plasmid were obtained. Tn917 serves as a selectable marker on these pLS20-derivatives and we can now select for transfer of the fertility plasmid. Restriction analysis of one of the marked plasmids, pXO503, and hybridization of the digest with the transposon vector indicate that Tn917 inserted into the 4.1-kb EcoRI fragment of pLS20. This plasmid has been transferred from B. subtilis to B. anthracis by mating. The ability of pXO503 to promote transfer of the resident B. anthracis plasmids is currently under investigation.

Plasmids other than pBC16 are being tested for pLS20-mediated transfer. Results presented here demonstrate that pLS20 mediates transfer of the Staphylococcus aureus kanamycin resistance plasmid pUB110 at frequencies comparable to those of pBC16 transfer. Plasmids pBC16 and pUB110 are homologous except for the region occupied by their resistance determinants (25). Therefore, pLS20-mediated transfer of pUB110 was not unexpected. We have also observed cotransfer of the B. subtilis (natto) plasmid pLS19 with pBC16 in

intraspecific and interspecific matings. These results suggest that mating may be a mechanism for the natural dissemination of plasmids among B. subtilis (natto) strains.

Thus far, attempts to transfer the constructed plasmids pTV1 and pTV24 (41) in pLS20-mediated matings have been unsuccessful. We have also tested for transfer of the naturally-occurring Staphylococcus aureus plasmids pC194 (10) and pE194 (39). B. subtilis PSLI strains carrying pLS20 and either pTV1, pTV24, pC194, or pE194 were unable to transfer these plasmids to B. subtilis (natto), B. anthracis, or restriction-deficient B. subtilis strains.

Further work comparing transmissible and nontransmissible plasmids will aid in investigation of the mechanism of pLS20-mediated plasmid transfer. In conjugal transmission of plasmids, nonconjugative plasmids may be transferred by formation of a cointegrate structure with a conjugative plasmid. This process, termed conduction, is mediated by recombination between homologous sequences which exist on the two plasmids, or by the association of the two plasmids during the translocation of a transposable genetic element. Conduction of plasmids often results in transcipients which inherit plasmids that differ from the donor plasmids. Unlike conduction, transmission of plasmids by donation occurs without physical association of the two plasmids. Donation of a transmissible plasmid occurs without the benefit of sequences homologous to those of the donating plasmid and without acquisition of insertion mutations (8).

Preliminary evidence indicates that transfer of pBC16 occurs by donation, rather than conduction. Restriction analysis of pBC16 obtained from three different transcipients revealed no differences in plasmid size or restriction pattern when compared to pBC16 DNA from a B. anthracis strain which had acquired pBC16 by CP-51-mediated transduction (data not shown). Furthermore, pLS20-mediated transfer of pBC16 occurred at a high frequency. Because conduction depends on relatively rare interactions, high frequency transfer is usually not observed when this mechanism is employed (8).

Transfer of chromosomal genes by pLS20 has not been demonstrated. In E. coli, transfer of the chromosome by plasmid F occurs by conduction. F and the chromosome contain homologous transposable sequences which facilitate generation of F::chromosome cointegrates (8). To test for B. subtilis chromosome transfer, a donor strain carrying pLS20 and a strain carrying pX0503 were each tested for the ability to transfer the wild-type allele of the trpC2 marker. Matings

between B. subtilis 168-derived mutants did not result in transfer of the marker tested. We are currently testing the ability of pX0503 to transfer chromosomal DNA from donor strains carrying Tn917 insertions in the chromosome.

Demonstration of a fertility plasmid in B. subtilis (natto) is of interest because it makes possible the transfer of genetic material between Bacillus species which are not closely related. Development of this method of genetic exchange will facilitate the introduction of plasmid DNA into nontransformable Bacillus species by use of transformable fertile B. subtilis and B. subtilis (natto) strains as intermediates.

pLS20 is the first reported example of a fertility plasmid in a Bacillus species other than thuringiensis. Many Bacillus species contain a number of endogenous plasmids whose physiological roles have not been determined. It is possible that plasmids analogous to pLS20 and the conjugative B. thuringiensis plasmids are ubiquitous.

II. Investigation of phage TP-21 whose prophage is a plasmid

In order to realize the full potential of many of the technical advances in molecular biology, there must be sufficient capabilities in the area of classical genetics. Within the closely related Bacillus species, B. anthracis, B. cereus, and B. thuringiensis, transduction of chromosomal markers has been in use for considerable time (6, 9, 31-33, 40) and plasmid transduction has been more recent (28). Transformation, although reported for B. thuringiensis, is as yet too inefficient to be of much practical use at this time. Relatively recently B. thuringiensis has been found to harbor fertility plasmids which can transfer themselves as well as other plasmids (4, 6). Toward the goal of building a framework for genetic manipulation, the B. thuringiensis plasmid transfer system has been used with B. anthracis in our laboratory for the transfer of plasmids, foreign as well as native. Because of our success with this system, the reports from Aronson's laboratory (1-3, 23) of conjugative chromosomal transfer from B. thuringiensis subsp. kurstaki HD1-9 to B. cereus 569K, mediated by a 29-Mdal plasmid carried by HD1-9, was met with great interest. Since our laboratory had found that B. thuringiensis fertility plasmids function very well in B. anthracis it seemed likely that

they would mobilize chromosomal markers in B. anthracis if they could do so in B. thuringiensis.

When we tested B. thuringiensis strain HD1-9 as the donor in matings with B. cereus 569K auxotrophs as the recipient, we did, indeed, find prototrophic B. cereus transcipients. The number varied greatly among individual experiments. Frequently the HD1-9 donor culture would lyse spontaneously, and in broth matings the mating mixtures usually appeared to contain lysed cells. Some selective plates in crosses with HD1-9 donors and B. cereus 569 UM27-11 recipients appeared to have plaques in the faint background growth. Therefore, we performed experiments in U-tubes to determine whether cell to cell contact was required for chromosomal transfer. There was a reduced but significant frequency of transfer of chromosomal markers across the 0.45 μm HA Millipore filter (Table 7).

DNase had no effect on the number of prototrophic transcipients when added to mating mixtures (data not shown). To test for the possible involvement of phage, cell free lysates were made from cultures of B. thuringiensis strains HD1-9 and HD1-9 td1(pBC16) following treatment with mitomycin C or ultaviolet light as well as from spontaneously lysed cultures. Table 8 shows the titers of plaque-forming units (PFU) in the cell-free lysates assayed on B. cereus 569. A BHI broth culture of strain HD1-9 which lysed spontaneously had a phage titer higher than those of L broth cultures induced with mitomycin C or ultraviolet light. At least two plaque morphologies were present on some assay plates. The predominant plaque was small and turbid, the other plaque type was also small but it was clear.

Cell-free lysates were highly effective in transducing chromosomal markers. Plate transductions in which 0.1 ml of early stationary phase B. cereus recipient cells (auxotrophic and Str^r) were spread with 0.1 ml of cell-free lysate on minimal medium containing streptomycin (1 mg/ml) yielded up to 1.5×10^4 prototrophic transductants per ml. This is substantially higher than the number of prototrophic colonies obtained from broth matings and about equal to the highest number generated in filter matings. These lysates were tested for the inactivation of PFU and transducing particles by antiserum to B. thuringiensis temperate phage TP-12. This phage was isolated in our laboratory about 10 years ago from a mixed culture of several B. thuringiensis strains which included subsp. kurstaki. Antiserum to TP-12 reduced the PFU of an HD1-9 lysate about ten thousand-fold and lowered the

number of transductants per ml to a value not significantly different from the number of spontaneous revertants per ml (data not shown).

Tc^r derivatives of strain HD1-9 were prepared by phage CP-51-mediated transduction of pBC16 (28). The transductants were streaked for isolation several times in the presence of phage CP-51 antiserum and confirmed to be free of CP-51. One Tc^r transductant, HD1-9 td1(pBC16), was used to prepare cell-free lysates for tests to determine their ability to transduce pBC16. The lysates were able to transfer pBC16 to B. cereus 569 UM25-9 and 569K. The number of Tc^r colonies varied from 80 to 500 per ml. All of several Tc^r transductants examined for plasmid content by agarose gel electrophoresis contained pBC16 in addition to their resident plasmids. Nearly all contained another plasmid which co-migrated with the 29-Mdal plasmid of B. thuringiensis subsp. kurstaki HD1-9.

It seems highly unlikely that the majority of transducing particles carrying pBC16 should also contain the 29-Mdal plasmid. At the multiplicity of infection employed, cells that received a transducing particle containing pBC16 probably also received one or more PFU and became lysogenized. Thus, if the 29-Mdal plasmid is the transducing phage, it should be present in lysogens. B. cereus 569 lysogens isolated from the centers of turbid plaques obtained upon assaying HD1-9 lysates contained a plasmid co-migrating with the 29-Mdal plasmid from HD1-9.

Experiments in which we attempted to transduce pBC16 into B. anthracis with HD1-9 td1 lysates were unsuccessful. This, along with the fact that such lysates did not form plaques when assayed on B. anthracis UM44-1, indicates that B. anthracis is a poor host for the phage responsible for plasmid transduction in these lysates. However, in experiments which used HD1-9 td1(pBC16) cells as donors of pBC16 to B. anthracis UM44-1 in broth matings, numerous Tc^r transcipients were produced. These pBC16-containing transcipients contained other plasmids of B. thuringiensis HD1-9, but not the 29-Mdal plasmid. When tested as a donor of pBC16 to another B. anthracis strain a UM44-1 transcipient was found to be fertile.

It seems probable that both conjugal and transductional transmission of pBC16 takes place in mating mixtures containing HD1-9 donors and B. cereus recipients. To determine whether both modes of transmission are also involved in the transfer of chromosomal markers, experiments which examined the transfer of chromosomal markers in mating mixtures containing TP-12 antiserum

were performed. Table 9 shows the results of these experiments. In matings of B. thuringiensis HD1-9 or HD1-9 td1 donors with B. cereus 569K recipients, antiserum to TP-12 reduced the yield of prototrophic transcipients to the number of spontaneous revertants in the control. This indicates that transfer of chromosomal markers in these mating mixtures occurred only by transduction. When the same mating mixtures were spread on L agar with tetracycline, all mixtures with HD1-9 td1 as donor, both with and without antiserum, produced Tc^r transcipients. These results show that plasmid transfer can take place in the absence of phage mediated transfer.

We conclude from the results presented above that the transfer of chromosomal markers from B. thuringiensis subsp. kurstaki HD1-9 to B. cereus 569 recipients is mediated by transducing phage and not by a mating process mediated by a 29-Mdal plasmid as reported by Aronson, et al. (1-3, 23). We have identified two transducing phages carried by kurstaki HD1-9 and there is evidence that at least a third transducing phage may be present. The prophage of one of the transducing phages exists as a 29-Mdal pladmid; we have named this phage TP-21. The phage is antigenically cross reactive with phage TP-12 and antiserum to TP-12 inactivated plaque-forming units and prevented the transduction of chromosomal markers. However, TP-12 and TP-21 are not the same phage; TP-12 prophage has not been observed to exist as a plasmid, and the two phages have different host ranges and very different plaquing characteristics.

TP-21 lysogens. Most B. thuringiensis strains carry one or more phages. R. B. Reynolds (Ph. D. thesis, University of Massachusetts, Amherst, 1982), working in my laboratory, identified five unique phages carried by a polylysogenic strain. Thus, we had to isolate TP-21 and free it from other phages carried by strain HD1-9. At appropriate dilutions cell-free filtrates from cultures of kurstaki HD1-9 produced plaques of two different morphologies on soft agar lawns of B. cereus 569. The more abundant was a small turbid plaque. Colonies isolated from the centers of plaques were tested for phage. Those isolates which produced phage were all found to contain the 29-Mdal plasmid. Phage lysate from one of these isolates was used to purify TP-21 by single plaque isolation on lawns of B. cereus 569. After three cycles of purification B. cereus strains lysogenic for TP-21 were isolated and again found to contain the 29-Mdal plasmid.

Both HD1-9 and TP-21 lysogens of B. cereus 569 release a phage which produces a clear plaque on soft agar lawns of B. cereus 569. TP-21 lysogens are immune to this phage and it is neutralized by TP-12 antiserum to the same extent as TP-21 (99%). This phage has been designated TP-21c, a clear plaque mutant of TP-21. When subjected to analysis with several restriction endonucleases, DNA extracted from particles of the clear-plaque mutant gave restriction patterns indistinguishable from those of the 29-Mdal plasmid (prophage) extracted from B. cereus 569 TP-21 lysogens. Fig. 5 shows a schematic drawing of the results obtained with XbaI endonuclease. DNA isolated from TP-21 phage particles is probably linear; it migrates in electrophoretic agarose gels at a rate approximating that of bacterial chromosoal DNA. This is analogous to phage P1 of E. coli. P1 prophage exists as circular plasmid DNA, but phage particles contain linear DNA.

Transposition of Tn917 into the TP-21 genome. To better study the usefulness of TP-21 it seemed advantageous to mark the phage genome in a way that would allow direct selection of all lysogens. This has the further advantage of allowing the detection of all infected cells whether they are able to form a plaque or not. TP-21 does not plaque on the B. anthracis strains tested although lysogens could be isolated from strain 4229. The clear-plaque mutant, TP-21c, formed plaques only on strain 4229 but turbid lysis was demonstrated in spot tests of a few other strains. Phage marked with the erythromycin resistance transposon could be used conveniently to determine the host range of TP-21.

A TP-21-sensitive host carrying Tn917, B. cereus 569 UM20-1 (pLS20::Tn917, pXO12, pBC16), was lysogenized with the phage. Two independently isolated lysogens were examined and found to contain the 29-Mdal plasmid characteristic of TP-21 lysogens. In order to increase the likelihood of Tn917 transposition into the phage genome and to allow this event to take place in many independent clones, one of these lysogens was serially cultured in BHI broth containing erythromycin (0.05 µg/ml). These cultures were maintained by transferring 0.2 ml into 25 ml of fresh broth for several passages. After three days the culture was grown in broth containing 25 µg of erythromycin per ml to eliminate any cells which might have lost the transposon, and the culture filtrate was assayed for plaque-forming units. The titer was 1 X 10^9 PFU/ml, and this lysate was used to transduce B. anthracis 4229 R1 and B. cereus 569 to erythromycin resistance. B. anthracis

4229 R1 was transduced by a procedure similar to that which is used for the transduction of pBC16 by CP-51; i.e., the transducing lysate and recipient cells were spread on filter membranes placed on a nonselective medium. After an appropriate amount of incubation time to allow for phenotypic expression, the filters were than transferred to selective medium. Subsequently a new method was devised which is simpler. Transducing lysates and recipient cells were mixed together and adjusted to contain 0.1 µg of erythromycin per ml. The mixture was then incubated for 45 min with shaking to allow the phage to adsorb. The low concentration of erythromycin induces resistance in cells containing Tn917, but it is not inhibitory to those cells without the transposon. After this period of incubation 0.2 ml of the transducing mixture was added to 2.5 ml of soft agar containing 0.1 µg of erythromycin per ml and this was poured on an agar plate containing selective levels of antibiotic (2 µg of erythromycin and 25 µg of lincomycin per ml). Colonies of tansductants could be seen after 16 h with B. cereus and after 24 h with B. anthracis.

The frequency of transduction (about 7×10^{-6} transductants per PFU) was the same by both methods and with both species. The transductants of each species were immune to TP-21c in spot tests. However, most isolates appeared to contain defective phage and were unable to produce plaque-forming units. Of several examined, only three B. anthracis 4229 R1 and six B. cereus erythromycin-resistant TP-21 immune isolates were able to produce phage.

To test whether the transposon had integrated into the phage genome, lysates from the nondefective lysogens were assayed on B. cereus 569 in soft agar lawns containing inducing but subinhibitory concentrations of erythromycin. Plaques were picked to plates containing selective concentrations of the antibiotic to determine whether the lysogens formed in these plaques were resistant to erythromycin. All of the B. anthracis erythromycin-resistant TP-21 lysogens produced phage which converted essentially every newly generated lysogen to erythromycin resistance. Four of the six B. cereus 569 erythromycin-resistant TP-21 lysogens, like the B. anthracis lysogens, were able to produce phage which presumably contained Tn917.

Agarose gel electrophoresis of plasmid extracts of the B. anthracis and B. cereus erythromycin-resistant transductants revealed newly acquired plasmid DNA which in size was equal to, larger than, or smaller than that of wild-type TP-21 DNA regardless of whether it was produced by a defective or a

nondefective lysogen. This demonstrates that some of the phage genomes acquired deletions as well as the transposon, either of which may have contributed to the defective character exhibited by the majority of lysogens. The insertion of Tn*917* into the phage genome may not inactivate any functions if, like coliphage P1, TP-21 is terminally redundant and circularly permuted. However, the acquisition of a deletion by a viable phage demonstrates that the wild-type TP-21 genome contains some nonessential DNA.

Determination of the host range of TP-21::Tn*917*. To determine which strains are sensitive to TP-21, a Tn*917*-marked derivative was used to select cells which had acquired erythromycin resistance and therefore also TP-21. Recipient cells and phage lysate were incubated together under erythromycin resistance-inducing conditions and then plated in soft agar on selective plates containing erythromycin and lincomycin. The results of this survey are shown in Table 10.

All of the eleven *B. anthracis* strains tested, with the exception of ΔAmes-1, were sensitive to TP-21. Strain 4229 was the most sensitive of the *B. anthracis* strains tested. *B. cereus* 569, as well as a TP-21 lysogen of 569 were sensitive, as expected. However, *B. cereus* strain T appeared to be insensitive. All three of the *B. thuringiensis* strains tested were sensitive. In more recent tests a number of other *B. thuringiensis* strains were tested and the majority of them were sensitive (data not shown). Thus, TP-21 appears to have an unusually broad host range.

Some of the strains yielded lysogens (transductants) at a very low frequency. It seems likely that in those cases either most of the cells were resistant and only sensitive mutants were selected or that the cells were resistant and host-range mutants of the phage were selected. If either of these possibilities is true, then it is likely that those strains which appeared to be completely resistant might in future tests yield transductants. These two possibilities could be distinguished experimentally, but the experiments have not yet been done. Nevertheless, examination of a number of transductants, including some of those from the relatively insensitive strains, showed that they contained the 29-Mdal plasmid (prophage).

Fig. 6 is a schematic representation of an agarose gel showing the presence of the 29-Mdal plasmid in cells lysogenized with TP-21 or TP-21::Tn*917*.

Defective TP-21::Tn917. During the process of isolating Tn917-tagged TP-21, several lysogens carrying defective phage were isolated. These defective lysogens do not form infectious TP-21, but the defective phages are maintained as plasmids. They encode MLS resistance and express immunity to lysis by the clear plaque mutant TP-21c. These defective phage may prove useful in determining the essential and nonessential regions of the TP-21 genome.

If the defective phage fails to form plaques as a result of the inability to produce some diffusible product, a cell infected with both mutant and wild-type phage should liberate a mixture of the two since the wild-type phage should be able to complement the defective phage. This is generally accomplished by superinfecting a derepressed defective lysogen with wild-type phage. TP-21 lysogens are not subject to derepression by either UV light or mitomycin C, but moderately high phage titers are obtained spontaneously following growth at 30°C for 16 h in BHI broth with 0.1% glycerol. In a preliminary test to determine the feasibility of this strategy a culture of B. anthracis 4229 R1 containing a TP-21::Tn917 defective prophage was grown with TP-21 helper phage. Cell-free filtrates from this experiment were assayed and essentially no phage was recovered. These results may not represent the interaction of all of the defective phage isolates with wild-type TP-21 and other isolates will be tested for complementation.

The bacteriophage CP-51, a generalized transducing phage for B. anthracis, B. cereus and B. thuringiensis, has been shown to transduce antibiotic resistance plasmids. Since the defective TP-21 prophages exist as plasmids, CP-51 was tested for the ability to transduce defective TP-21::Tn917. CP-51 was propagated on B. cereus 569 lysogens harboring defective TP-21 and the lysates were used to test for the transfer of MLS resistance to B. thuringiensis HD1-9 by plasmid transduction. B. thuringiensis HD1-9 is the parent strain lysogenic for TP-21 and it was used as a recipient in order to examine the effect of the defective prophage on the natural host of TP-21. Representative erythromycin-resistant transductants were isolated and purified. When the transductants were examined for plasmid content the HD1-9 resident 29-Mdal plasmid was found to have been replaced by a slightly smaller plasmid the size of the defective prophage contained in the donor. This demonstrates that the incompatibility region of the TP-21 prophage was functional in these mutants. The erythromycin-resistant HD1-9

transductants no longer produced TP-21 but they did continue to produce the other temperate phage carried by the parent strain. These HD1-9 defective lysogens should be of great help in the examination of the other temperate phage carried by this strain.

Attempts to isolate TP-21::Tn917 mutants which are temperature sensitive for replication. We are very interested in obtaining a mutant of TP-21::Tn917 which is temperature sensitive for replication. Such a mutant would be a valuable and powerful tool for transposon mutagenesis. Because the phage has such a wide host range, it would be useful for transposon mutagenesis in B. anthracis, B. cereus, and B. thuringiensis. We are now concentrating on the isolation of such mutants. Our first attempts have been unsuccessful but I am optimistic that we should be able to obtain such mutants. Generally phage mutants that are temperature-sensitive for replication are relatively easy to isolate. Our problem is somewhat complicated by the fact that TP-21 plaques very poorly. We are attempting to find better conditions for plaque formation.

In our search for temperature-sensitive mutants we will also screen for mutants that are temperature inducible. The availability of such mutants would simplify the problem of propagating TP-21.

III. Physical and genetic characteristics of the Bacillus thuringiensis subsp. thuringiensis fertility plasmids pXO11 and pXO12

We reported previously on the identification of two self-transmissible plasmids, pXO11 (56 Mdal) and pXO12 (75 Mdal), indigenous to B. thuringiensis 4042A subsp. thuringiensis. Restriction analysis of two crystalliferous (Cry^+), transfer deficient (Tra^-) deletion derivatives of pXO12 has shown that gene(s) responsible for conjugal transfer functions are located on a 48.5-kb AvaI fragment. DNA-DNA hybridization experiments have shown that a copy of the B. thuringiensis transposon, Tn4430, is located on a 5.7-kb BglII fragment contained within the 48.5-kb AvaI fragment of pXO12. Analysis of two acrystalliferous (Cry^-), transfer proficient (Tra^+), erythromycin resistant (Em^r) derivatives of pXO12 has indicated that a 16.0-kb AvaI fragment encodes information required for the synthesis of parasporal crystals, and the 16.0-kb and the 48.5-kb AvaI fragment are adjacent on pXO12. Based on these results,

it appears that Tn4430, the transfer genes, and the parasporal crystal gene are located in the same vicinty on pXO12. DNA-DNA hybridization experiments have shown that the homology exhibited by the four conjugative B. thuringiensis plasmids pXO11, pXO12, pXO13 and pXO14 can be attributed in part to the presence of Tn4430. The transfer efficiency of pXO12 has been determined by using marked derivatives of pXO12 which harbor the Em^r transposon Tn917; transfer frequencies ranged from 9.2×10^{-5} to 7.1×10^{-2}.

Restriction analysis of pXO12 plasmid derivatives. One approach to mapping the transfer gene(s) of pXO11 and pXO12 is by restriction analysis of transfer deficient (Tra^-) derivatives of these plasmids. Comparative analysis of Tra^- and wild type Tra^+ plasmids should permit the preliminary assignment of conjugal transfer function(s) to specific restriction fragrent(s) on these plasmids. Due to the availability of both partially and completely transfer deficient pXO12 deletion derivatives, we have decided to first concentrate on mapping the transfer gene(s) of pXO12 and then use this information to examine the transfer region(s) of pXO11.

Previous restriction analysis of two independently isolated Tra^- Cry^+ pXO12 deletion derivatives (pXO12Δ43 and pXO12Δ203) revealed that both mutants have undergone a 20- to 25-kb deletion in the following restriction fragments: (1) 70.0-kb PstI fragment, (2) 59.8-kb ClaI fragment, and (3) 48.5-kb AvaI fragment. These results suggest that these three restriction fragments encode gene(s) required for conjugal transfer ability. To delineate more precisely the location of the transfer genes on pXO12, the smallest restriction fragment (48.5-kb AvaI fragment) was chosen for further genetic and physical analysis.

Restriction analysis of two Cry^-, Tra^+), Em^r pXO12 plasmid derivatives (pXO12-706 and pXO12-730) has indicated that a 16.0-kb AvaI fragment encodes information required for the synthesis of parasporal crystals. A schematic representation of the AvaI restriction patterns of the pXO12 derivatives designated pXO12Δ43, pXO12Δ203, pXO12-706 and pXO12-730 is shown in Fig. 7.

To confirm the loss of the 48.5-kb AvaI fragment in the Tra^- Cry^+ deletion derivatives, pXO12Δ43 and pXO12Δ203, a blot of the AvaI digests of pXO12, pXO12Δ43, pXO12Δ203, pXO12-706 and pXO12-730 (Fig. 7) was probed for hybridization with ^{32}P-48.5-kb AvaI fragment of pXO12. The ^{32}P-48.5-kb AvaI fragment hybridized to the newly generated fragments on pXO12Δ43 and pXO12Δ203 (25.0, 23.6 and 14.0 kb) and to the unaltered 48.5-kb AvaI fragment of the Tra^+ Cry^- derivatives pXO12-706 and pXO12-730. The concomitant loss of the

48.5-kb AvaI fragment and the 16.0-kb AvaI fragment in the deletion derivative pXO12Δ203 suggests that these two fragments are adjacent to one another on pXO12.

A more detailed analysis of the 48.5-kb AvaI fragment using the restriction endonuclease BglII was undertaken for the following reasons. Identification of smaller BglII fragments contained within the 48.5-kb AvaI fragment of pXO12 could be useful in future Southern hybridization experiments and also in DNA cloning experiments. For example, subcloning various combinations of the BglII fragments generated by partial digestion of the 48.5-kb AvaI fragment may give rise to Tra^+ recombinant plasmids more readily than shotgun cloning of whole pXO12 DNA.

Schematic representations of the BglII restriction patterns of pXO12, the 48.5-kb AvaI fragment, pXO12Δ43 and pXO12Δ203 are shown in Fig. 8. Digestion of pXO12 with the restriction enzyme BglII gives rise to 21 restriction fragments ranging in size from 19.6 kb to 0.8 kb. There were 12 BglII fragments within the 48.5-kb AvaI fragment of pXO12. BglII restriction analysis of pXO12Δ43 and pXO12Δ203 revealed that both Tra^- Cry^+ deletion derivatives have lost the same 9 BglII fragments; 7 of the 9 fragments (8.7, 5.7, 4.2, 3.7, 3.3, 3.1 and 3.0 kb) were contained within the 48.5-kb AvaI fragment of pXO12. According to these results, information required for conjugal plasmid transfer may be more precisely mapped to 7 BglII fragments (representing approximately 31.7 kb) within the 48.5-kb AvaI fragment of pXO12.

Although the above results strongly suggest that the 48.5-kb AvaI fragment of pXO12 carries sequences of at least one gene required for conjugal plasmid transfer, the extent of the transfer region(s) contained within this fragment is presently unknown. In the hope of positively identifying one genetic element within the 48.5-kb AvaI fragment, we decided to probe this fragment with the recently discovered B. thuringiensis transposon Tn4430 (18) for the following reasons. Previous Southern hybridization experiments have shown that pXO12 harbors one copy of Tn4430. In addition, recent work in our laboratory has implicated this transposon in having a role in the transfer of large plasmids by pXO12. Therefore, it would be interesting to determine whether Tn4430 is located in the same vicinity as the transfer gene(s) on pXO12.

To determine whether Tn4430 is located within the 48.5-kb AvaI fragment of pXO12, BglII restriction digests of pXO12, the 48.5-kb AvaI fragment of pXO12, pXO12Δ43 and pXO12Δ203 were probed for hybridization with ^{32}P-labelled pHT44 (pUC18::Tn4430). The results of this DNA-DNA hybridization experiment are shown in Fig. 8. ^{32}P-labelled pHT44 hybridized to a 5.7-kb BglII fragment contained within the 48.5-kb AvaI fragment of pXO12. No positive signal was detected in the two Tra$^-$ Cry$^+$ deletion derivatives, pXO12Δ43 and pXO12Δ203, which have lost the 5.7-kb BglII fragment. The above results suggest that: (1), at least one or more transfer genes are located within the AvaI fragment of pXO12, and (2), the B. thuringiensis transposon Tn4430 is in close proximity to the transfer gene(s) on pXO12.

Molecular cloning of pXO12 DNA. One other approach to mapping the transfer gene(s) of pXO12 is shotgun cloning of partially digested pXO12 DNA in B. subtilis using multicopy antibiotic resistance plasmid vectors. Several shotgun cloning experiments have been performed using: (1) the tetracycline resistance plasmid pBC16 (2.8 Mdal), (2) the erythromycin and tetracycline resistance plasmid pTV24 (8.9 Mdal), and (3) the chloramphenicol and kanamycin resistance plasmid pBD64 (3.2 Mdal) as plasmid vectors. Most success in obtaining recombinant DNA molecules has come through the use of the plasmid vector pBD64; insertion of foreign DNA into it's unique BglII site inactivates the expression of the kanamycin resistance gene (11). However, thus far no Tra$^+$ recombinant plasmids have been detected with any of the vectors listed above.

Homology among the B. thuringiensis conjugative plasmids. In view of the ubiquity of large molecular weight self-transmissible plasmids among naturally occurring strains of B. thuringiensis, it would be interesting to determine the extent of homology among the transfer genes on these plasmids. Preliminary results from Southern hybridization experiments have shown that pXO11 shares considerable homology with pXO12 and two other B. thuringiensis conjugative plasmids, pXO13 and pXO14 (26). For these experiments pXO11 DNA was labelled with ^{32}P and then hybridized to nitrocellulose blots of PstI and EcoRI restriction digests of the B. thuringiensis conjugative plasmids, pXO11, pXO12, pXO13, and pXO14.

The large extent of homology observed among pXO11, pXO12, pXO13, and pXO14 may be attributable to DNA sequences unrelated to conjugal transfer functions such as: (1) parasporal crystal genes, (2) inverted repeat

sequences, and (3) transposon-like elements (17, 20-22). In fact, subsequent DNA-DNA hybridization experiments have revealed that all four of the conjugative plasmids harbor one copy of the B. thuringiensis transposon Tn4430 (Fig. 9). For this experiment, pHT44 (pUC18::Tn4430) was radiolabelled with ^{32}P and then hybridized to nitrocellulose blots of PstI restriction digests of the four plasmids. Lereclus et. al. (21) have determined that Tn4430 harbors an internal PstI site. Therefore, the presence of Tn4430 on the PstI-cut plasmids should result in the hybridization of two fragments. As expected, two PstI fragments showed homology to ^{32}P-pHT44 for each plasmid tested.

Due to the molecular relatedness between B. thuringiensis plasmids in general (18), whole plasmid DNA is not a good probe for specifically determining the extent of homology among the transfer genes on these plasmids. Analysis of pXO12 and the Tra$^-$ Cry$^+$ deletion derivatives, pXO12Δ43 and pXO12Δ203, with the restriction endonuclease AvaI has indicated that the 48.5-kb AvaI fragment of pXO12 carries gene(s) required for conjugal transfer ability. This putative Tra$^+$ restriction fragment should provide a more specific probe for examining the molecular relatedness of the transfer genes on pXO11, pXO12, pXO13 and pXO14.

A blot of the BglII restriction patterns of pXO11, pXO12, pXO13 and pXO14 was probed for hybridization with the ^{32}P-48.5-kb AvaI fragment. The results of this hybridization experiment are shown in Fig. 10. As expected, 12 BglII fragments of pXO12 hybridized to the ^{32}P-48.5-kb AvaI fragment. For the plasmids pXO11, pXO13 and pXO14, the ^{32}P-48.5-kb AvaI fragment hybridized with 2 and 3 BglII fragments, respectively.

A blot of the BglII restriction bands of pXO11, pXO12, pXO13 and pXO14 was also probed for hybridization with ^{32}P-labelled pHT44 (pUC18::Tn4430) and the results from this experiment are included in Fig. 10. ^{32}P-pHT44 hybridized to one BglII fragment on each of the four conjugative plasmids. For each plasmid, the particular fragment exhibiting a positive signal when probed with ^{32}P-pHT44 also showed homology to the 48.5-kb AvaI fragment of pXO12. The fact that all four plasmids exhibited one or more distinct fragments that were homologous to the 48.5-kb AvaI fragment but not homologous to Tn4430 suggests that pXO11, pXO12, pXO13 and pXO14 harbor other homologous genetic elements, i.e., transfer gene(s) and insertion-like elements.

Genetic marking of pXO12 with Tn917. In contrast to the antibiotic resistance and bacteriocin determinants of other gram-positive and

gram-negative conjugative plasmids, no readily selectable phenotypes have been assigned to any of the B. thuringiensis conjugative plasmids. To date, the only plasmid-encoded functions for some but not all B. thuringiensis plasmids are the cryptic transposon Tn4430 and the production of an insecticidal toxin known as the delta-endotoxin or parasporal crystal. Although parasporal crystals are easily detected by phase contrast microscopy, this does not provide a selectable trait for plasmid transfer. To overcome this problem, transfer of the B. thuringiensis conjugative plasmids studied in our laboratory has been monitored indirectly, i.e., by the transfer of the tetracycline resistance plasmid pBC16.

However, genetic marking of the B. thuringiensis conjugative plasmids, is highly desirable since it would enable us to monitor their transfer directly. Transposition of the erythromycin resistance transposon Tn917 into these plasmids would provide a genetic marker for these plasmids.

A "mating out" assay was designed to construct B. anthracis and B. cereus strains harboring pXO12::Tn917 derivatives. For this procedure pXO503, a derivative of the B. subtilis (natto) conjugative plasmid, i.e., pLS20::Tn917, was used as a transposition vector. To construct pXO12::Tn917 derivatives, a mating was performed between the donor B. anthracis Weybridge UM44-1 tr203-23(pXO1, pXO12, pBC16) and the recipient B. anthracis Weybridge A UM23C1-2 tr135K-1 (pXO503). A transcipient designated B. anthracis Weybridge A UM23C1-2 tr689B-3 was isolated which, according to agarose gel electrophoresis, contained the plasmids pXO12, pXO503, and pBC16.

To induce transposition of Tn917 onto pXO12, the transcipient tr689B-3 was grown in the presence of an inducing concentration of erythromycin (20 ng/ml) and mitomycin C (10 ng/ml) (38) and the induced culture was used to prepare mating mixtures with B. cereus 569 UM20-1. The rationale was that matings in broth would favor the transfer of pXO12 over that of the pLS20 derivative pXO503. Previous work in our laboratory has indicated that in contrast to pXO12, pLS20 mediates the transfer of itself and other Bacillus plasmids more readily on solid medium than in broth. Therefore, it would be likely that Str^r Em^r Tet^s transcipients derived from induced B. anthracis Weybridge A UM23C1-2 tr689B-3 (pXO12, pXO503, pCB16) donors would inherit pXO12::Tn917 derivatives.

An average of 5.4×10^2 Str^r Em^r Tet^s B. cereus transcipients per ml were derived from the induced B. anthracis donor. Plasmid analysis of several of

the transcipients revealed that they had inherited a plasmid slightly larger than the native pXO12. Mating experiments with the putative pXO12::Tn917^{+} B. cereus transcipients as donors indicated that they were effective donors of the Em^{r} marker to B. anthracis recipients.

Physical characterization of two Tn917-labelled derivatives of pXO12 designated pXO12-706 and pXO12-730 from B. anthracis Weybridge A UM23C1-2 tr706B-5 and B. cereus 569 U M20-1 tr730B-14, respectively, has been carried out. AvaI restriction analysis was performed to confirm the acquisition of Tn917 DNA in pXO12-706 and pXO12-730. Perkins and Youngman (24) have found that Tn917 has four cleavage sites for AvaI. Two of the sites are 7 base pairs from the ends of the terminal inverted repeats. Therefore, digestion with AvaI would permit unambiguous identification of Tn917 on pXO12::Tn917 derivatives by essentially excising the entire transposon into three distinct restriction fragments (2.2, 1.8 and 1.2 kb). Results of our analysis showed that pXO503, pXO12-706 and pXO12-730 all released 2.2-, 1.8- and 1.2-kb fragments upon digestion with AvaI. These data confirmed the presence of Tn917 on these three plasmids.

To determine the transfer efficiency of pXO12 by direct selection, matings were performed between B. anthracis and B. cereus donors carrying the transposon-tagged derivatives of pXO12 and erythromycin sensitive B. anthracis and B. cereus recipients. The results of these mating experiments are shown in Table 11. The transfer efficiency of pXO12 (as measured by the transfer of Em^{r}) was comparable to that of pXO12-mediated transfer of pBC16; transfer frequencies ranged from 9.5×10^{-5} to 7.1×10^{-2} with B. anthracis and B. cereus recipients.

IV. Transfer of pXO1 and pXO2 by the B. thuringiensis fertility plasmid pXO12

Plasmid pXO12, is able to mediate the transfer of pXO1 and pXO2 into B. cereus and cured B. anthracis recipients. In contrast to the relatively high frequency of pBC16 transfer, the number of transcipients which acquired the B. anthracis plasmids was significantly lower. In several cases a 4.2-kb DNA sequence originating from pXO12 was found to have transposed onto pXO1 and pXO2 after transfer. This 4.2-kb sequence was determined to be identical to the recently described B. thuringiensis transposon Tn4430. Transcipients were also obtained which harbored cointegrate plasmids of pXO1 and pXO12 which were capable of transferring the PA^+ (protective antigen) phenotype of pXO1 to 50% of the total Tc^r transcipients.

Transfer of pXO1 mediated by pXO12. To demonstrate the ability of the B. thuringiensis fertility plasmid pXO12 to mobilize pXO1, several strains of B. anthracis which contained pXO12, pBC16, and pXO1 were used as donors in matings with cured strains of B. anthracis. Transcipients were selected for the acquisition of pBC16, and screened for their ability to produce halos on immunoassay agar. As shown in Table 12, pXO12 was able to mobilize pXO1. In contrast to the relatively high frequency transfer of pBC16, the number of transcipients which acquired pXO1 was quite low. Examination of the plasmid content of the presumed pXO1-containing transcipients revealed several plasmid profiles (Fig. 12). While some transcipients inherited plasmids which could not be distinguished from the parent plasmids, others apparently acquired recombinant or cointegrate plasmids of pXO1 and pXO12.

Isolation of a $PA^{\pm}$ $Cry^{\pm}$ Tra^{-} transcipient containing a recombinant plasmid of pXO1 and pXO12. Examination of the plasmid profile of one of the PA^+ transcipients obtained in mating 156G (Weybridge UM44-1 C9 tr156G-2) showed that it harbored a single plasmid migrating in electrophoretic gels above chromosome DNA approximately the size of pXO12. In addition to its ability to produce protective antigen, this transcipient was Cry^+ but unable to mobilize pBC16. To determine if this plasmid was a recombinant of pXO1 and pXO12 the transcipient was cured of pBC16 by incubation at 42C to generate UM44-1 C9 tr156G-2 H1. Plasmid DNA was then extracted and analyzed by EcoR1 and Pst1 digestion. The restriction profile was compared to similarly digested pXO1 and pXO12 DNA. By both EcoR1 and Pst1 digestion the plasmid from tr156G-2 (designated pXO1-pXO12 for the present) was shown to contain

both pXO1 and pXO12 DNA. By EcoR1 restriction digestion analysis pXO1-pXO12 contained approximately 107 kb, consisting of 40.5-65.5 kb of pXO1 DNA and 41.5-66.5 kb of pXO12 DNA. Without a restriction profile map of pXO1 and pXO12 however, it is not possible to determine exactly how this plasmid was formed.

Isolation of cointegrate plasmids of pXO1 and pXO12. In several independent matings transcipients were obtained which inherited plasmids that were larger than pXO1 and pXO12 suggesting the formation of cointegrate molecules. Examinaton of two other UM44-1 tr156G transcipients, tr156G-5 and tr156G-6, revealed that each of them harbored two plasmids migrating in electrophoretic gels above chromosomal DNA, one slightly above and one below the position of pXO1. When these strains were used as donors in matings with B. anthracis UM23 C1-2 approximately 50% of the Tc^r transcipients acquired the ability to produce protective antigen (see matings 169G and 170G, Table 12). To determine whether either or both of the large plasmids were transferred to B. anthracis UM23 C1-1 in mating 169G, we examined 12 PA^+ and 4 PA^- 169G transcipients for their plasmid content. In every case the PA^+ transcipients appeared to contain only the larger of the two plasmids migrating above chromosomal DNA, while the PA^- transcipients were devoid of plasmid DNA migrating above the chromosomal DNA. In addition all of the PA^+ transcipients were Cry^+ while all of the PA^- transcipients examined were Cry^-. From these results it seems probable that a cointegrate plasmid had been formed between pXO1 and pXO12.

Examination of the plasmid profile of transcipients from mating 220G again suggested that cointegrate plasmids of pXO1 and pXO12 had been formed in the course of transfer. Examination of the plasmid profile of the PA^+ Cry^+ transcipients from mating 220G showed that 4 of the 5 transcipients harbored a single plasmid above the chromosome which was larger than both pXO12 and pXO1. As in the case of the 156G transcipients, UM23 C1-2 tr220G-2 and tr220G-4 were also able to transfer the PA^+ phenotype at very high frequencies (see matings 229G and 231G, Table 12).

Restriction analysis of pXO1.3 from B. anthracis UM23 C1-2 tr201G-1. Examination of the plasmid profile of B. anthracis UM23 C1-2 tr201G-1, obtained from a mating between B. anthracis UM2 tr244-1 and B. anthracis UM23 C1-2 (Table 12), showed that it contained a single plasmid migrating above the chromosome. To determine whether this plasmid was pXO1 and whether any

alterations had occurred as compared to pXO1.1 from the donor strain, the transcipient UM23 C1-2 tr201G-1 was cured of pBC16 by incubation at 42°C to isolated the preseumed pXO1 plasmid biologically. This generated strain UM23 C1-2 tr201G-1H3. Plasmid DNA extracts from the strain were digested with EcoR1, Pst1, and Kpn1 and subjected to gel electrophoresis. The digested DNA was compared to similarly digested pXO1 from B. anthracis UM2 (considered to carry wild-type pXO1) and pXO1.1 from B. anthracis UM2 tr244-1 CN3H2. Examination of the restriction profile of the plasmid from UM23 C1-2 tr201G-1H3 revealed that it was in fact a pXO1 derivative (Fig. 13), from hereon designated pXO1.3). The results also suggested that pXO1.3 had acquired the presumed transposon from pXO12. When the plasmid was digested with Kpn1 a fragment of 4.2 kb was generated which was not present in pXO1.1. This was presumably the same fragment present in pXO1.2 from the high-frequency donor reported previously, UM23 C1-1 tr47G-34N2 (34, 36).

It has been shown that pXO1.1 in the donor strain B. anthracis UM2 tr244-1 acquired 2.65-2.8 kb of pXO12 DNA. By restriction digestion analysis we have determined that the 5.4 kb Pst1 fragment of wild-type pXO1 was replaced by a fragment of 8.2 kb. Similarly, the 7.95 kb EcoR1 fragment which is present in wild-type pXO1 was replaced by two additional fragments of 6.45 and 4.15 kb. The toxin plasmid showed further changes in the high-frequency donor reported previously (34, 36) and referred to above, transcipient B. anthracis UM23 C1-1 tr47G-34. This altered derivative of pXO1 is designated pXO1.2. There was a loss of the 8.2 kb Pst1 fragment, a gain of two new fragments of 7.5 and 4.5 kb, and the 6.45-kb EcoR1 fragment was replaced with a fragment of 10.6 kb. There was no change in the 4.15-kb EcoR1 fragment seen in the low-frequency donor. In both the Pst1 and EcoR1 digests the alterations in pXO1.2 in the high frequency donor resulted in an additional 3.8-4.15 kb of DNA over that seen in pXO1 from the low frequency donor, or a total of 6.6-6.9 kb additional DNA as compared to wild-type pXO1. Schematic representations of the restriction fragment profiles of pXO1.1 from UM2 tr244-1 and pXO1.2 from UM23 C1-1 tr47G-34 are shown in Fig. 14. Comparison of the Pst1 and EcoR1 digests showed that the second alteration in pXO1.3 from UM23 C1-2 tr201G-1 was in a different region of the toxin plasmid as compared to pXO1.2 from UM23 C1-1 tr47G-34. Whereas the fragments unique to pXO1.1 as compared to pXO1 were in turn altered in pXO1.2 from UM23 C1-1 tr47G-34 CN3 (see Fig. 14), these fragments remained unchanged in pXO1.3 (Fig. 13). As

shown in Fig. 13 the 8.2-kb Pst1 fagment and the 6.45-kb and 4.15-kb EcoR1 fragments were unchanged in pXO1.3. The change in pXO1.3 involved the 5.5-kb EcoR1 fragment which was replaced with a 9.8-kb fragment. The alteration was not as clear when pXO1.3 was digested with Pst1. There did appear to be some changes in the large fragments and a 5-kb fragment, appearing now as a doublet, was generated.

pXO12 DNA is present on pXO1 plasmids after transfer. We reported previously (36) that the altered fragments in pXO1.1 and pXO1.2 showed homology to pXO12. To confirm that pXO1.3 had also acquired pXO12 DNA, and that the changes in pXO1.3 were not a result of the rearrangement of DNA sequences on pXO1.1, DNA from the agarose gel represented in Fig. 12 (lane 8, pXO1.3) was transferred to a GeneScreen Plus nylon membrane and probed with ^{32}P-dGTP-labelled pXO12 DNA. As indicated in Fig. 13 pXO12 showed strong homology to the restriction fragments unique to pXO1.3.

pXO12 and pXO1 derivatives contain transposon Tn4430. The mobilization of nonconjugative plasmids can occur by either conduction or donation. By definition, mobilization by conduction requires physical contact between the fertility plasmid and the plasmid being mobilized, while donation does not require physical contact of the two plasmids. In general the frequency of mobilization of nonconjugative plasmids by conduction is quite low when compared to the transfer frequency of the fertility plasmid. Because of the low number of transcipients which acquire pXO1 or pXO2 in the course of a mating it is believed that mobilization of the B. anthracis plasmids occurs by conduction.

Several investigators have identified transposons and insertion sequences on the chromosomes and plasmids of a variety of subspecies of B. thuringiensis. In one case a transposon from a 42-Mdal plasmid present in kurstaki KT$_o$ was found to have transposed onto the Streptococcus faecalis plasmid pAMβ1 in the course of a mating (20). This transposon has subsequently been designated Tn4430. To determine whether the pXO12 DNA on pXO1 derivatives was related to this transposon, we obtained the plasmid pHT44 from Marguerite-M. Lecadet. pHT44 was constructed by inserting the internal 4.2-kb Kpn1 fragment (which represents all but 2 bp at each end of the transposon) into the Kpn1 site of the Escherichia coli cloning vector pUC18. To determine the relatedness of the elements, plasmids pXO1, pXO1.1, and pXO1.2 were digested with the restriction endonucleases EcoR1, Pst1, and Kpn1,

electrophoresed in 0.7% agarose gels and transferred to GeneScreen Plus nylon membranes. Plasmid pHT44 which had been extracted from E. coli JM83 and labelled with [^{32}P]-dGTP was then used to probe the pXO1 plasmid DNA. As expected, pHT44 hybridized only to those fragments of pXO1 which had acquired the 4.2-kb pXO12 sequences.

To determine whether the element from pXO12 was identical to Tn4430 we isolated both elements and compared the DNA fragments generated after digestion with the restriction endonucleases Pst1 and HindIII. The elements were obtained by restricting pXO12 and pHT44 with Kpn1 and electrophoresing the DNA on 0.7% agarose gels. The 4.2 kb Kpn1 fragment was isolated from the agarose gels using an Elutrap apparatus as described by the supplier (Schleicher and Schuell). Approximately 0.5 µg of each DNA fragment was digested with either Pst1 or HindIII and the DNA was electrophoresed in a 1.2% agarose gel. As reported by Lecadet, at al. (38), the 4.2-kb Kpn1 fragment contains a single recognition site for each of the endonucleases Pst1 and HindIII which results in two fragments after digestion. The results of the digestion (Fig. 15) show that the 4.2 kb element from pXO12 is identical to Tn4430. Digestion of each fragment with HindIII produced fragments of 3.5 kb and 0.7 kb. Similarly when the fragments were digested with Pst1 two fragments of 3.1 kb and 1.1 kb were generated.

Plasmid pBC16 is unaltered after transfer. The difference in the frequency of mobilization of pBC16 and the large B. anthracis plasmids suggests that there may be two separate mechanisms of transfer. The high frequency transfer of pBC16 suggests that mobilization of this small plasmid occurs by donation. By using a pXO12 plasmid which was tagged with Tn917 (see above) we have determined that the transfer of pBC16 reflects very accurately the actual transfer frequency of pXO12. By definition, mobilization of plasmids by donation does not require physical contact between the conjugative plasmid and the nonconjugative plasmid. Accordingly there should not be any alteration in the nonconjugative plasmid after it has been transferred. To confirm that this was the case with pBC16 we examined the plasmid before and after mobilization by pXO12. Plasmid DNA was extracted from B. anthracis UM23 C1 td1(pBC16) and B. anthracis UM23 C1-2 tr272G-2(pBC16) and digested with EcoR1, for which pBC16 has a single recognition site, and electrophoresed in a 1.2% agarose gel. Examination of the gel showed that there was no change in the restriction pattern of pBC16 after it was mobilized by pXO12. This result

strengthens the idea that mobilization of pBC16 by the fertility plasmid pXO12 occurs by donation. It was possible that homologous sequences between pXO12 and pBC16 accounted for the ability of pBC16 to be transferred, though the high number of transcipients strongly suggests donation. To test the possibility that there is homology between pBC16 and pXO12, DNA from the agarose gel described above was transferred to GeneScreen Plus nylon membranes and probed with [^{32}P]-dGTP-labelled pXO12 DNA. Results of the hybridization experiment confirmed that there was no homology between the two plasmids.

pXO12 from B. anthracis pXO1 donor strains is unchanged as compared to wild type pXO12. Because transposons generally leave a copy of themselves at their original location when they transpose, there should not be any change in pXO12 from pXO1 donors or transcipients as compared to wild-type pXO12. To confirm this, pXO12 was isolated from UM2 tr244-1 and UM44-1 C9 tr41G-1, transcipients which carried pXO12 and pXO1, and from the transcipient B. cereus 569 UM20-1 tr374B-5, which contained only pXO12 and pBC16. DN. Digestion of the plasmid DNAs with EcoR1 revealed no detectable differences in the various pXO12 plasmids. These results are consistent with the hypothesis that there has been transposition of pXO12 sequences onto the toxin plasmid of B. anthracis.

Plasmid pXO2 sometimes contains Tn4430 after mobilization by pXO12. To confirm further the role of Tn4430 in the mobilization of the B. anthracis plasmids we examined the capsule plasmid pXO2 before and after mobilization by pXO12. To examine the plasmids it was first necessary to isolate pXO2 from the Cap$^+$ transcipients which also contained pXO12 and pBC16. This was done by using the transducing bacteriophage CP-51ts45. Transducing lysates were obtained by propagating the bacteriophage on the transcipients B. anthracis 4229 tr299-3, B. cereus 569 M20-1 tr305-1, and 569 UM20-1 tr305-5 and transducing B. anthracis UM23 C1-1 to Cap$^+$. In this way B. anthracis UM23 C1-1td20G, td22G, and td25G were obtained which contained pXO2 from tr299-3, tr305-5, and tr305-1, respectively. Plasmid DNA from the Cap$^+$ transductants was digested with the restriction endonucleases EcoR1 and Kpn1 and compared to similarly digested pXO2 DNA from B. anthracis 4229 which was taken to be the wild type. No alteration was detected in pXO2 which originated from B. anthracis 4229 tr299-3 as compared to the wild-type capsule plasmid. However, when pXO2 from the transcipient B. cereus 569 UM20-1 tr305-1 was digested with

Kpn1 a new fragment of 4.2 kb was generated suggesting the acquisition of Tn4430. Interestingly, when pXO2 from B. cereus 569 UM20-1 tr305-5, a transcipient from the same mating as tr305-1, was examined there was no indication that Tn4430 was present.

It seemed posssible that in the case of pXO2 transfer into B. cereus 569 UM20-1 tr305-5 there was generation of a cointegrate in the mating process but the transposon was lost from the pXO2 plasmid upon establishment in the transcipient strain. If this was the case, it seemed possible that if 569 UM20-1 tr305-5 was used as a donor to transfer pXO2, the capsule plasmid in Cap$^+$ transcipients might acquire Tn4430. To test this hypothesis B. cereus 569R UM20-1 tr305-5 was mated with B. anthracis UM23C1-2 and Cap$^+$ transcipients were selected. Cap$^+$ transcipients were obtained in this mating at approximately the same frequency as in matings with the original donor B. anthracis 4229 tr299-3. Plasmid pXO2 DNA was isolated from one of the transcipients and examined for alterations. When the restriction fragment profile of this plasmid was examined with EcoR1 and Pst1, again there was no alteration in the plasmid as compared to wild type pXO2. In Southern hybridization experiments there was no evidence of homology between pXO2 and pXO12, ruling out the possibility of homologous DNA sequences mediating contact of the two plasmids.

Discussion. By definition, transfer by conduction requires physical contact between the conjugative plasmid and the plasmid being mobilized. Evidence for the physical contact between pXO12 and the B. anthracis plasmids, pXO1 and pXO2, was two-fold. Examination of pXO1 and pXO2 after being transferred showed that in some instances they had acquired a 4.2-kb DNA sequence. This sequence was found to have originated from the fertility plasmid pXO12, and by Southern hybridization and restriction digestion analysis was determined to be identical to the recently described B. thuringiensis transposon Tn4430. Restriction analysis of pXO12 from a donor and a transcipient revealed no alterations in that plasmid, a result consistent with the presumed transposition of Tn4430 from pXO12 to pXO1 and pXO2. It should be emphasized, however, that pXO12 DNA was not always found in pXO1 and pXO2 following their transfer. If mobilization of the B. anthracis plasmids does in fact require physical contact with pXO12, presumably the transferred complex frequently resolves in such a way that pXO1 and pXO2 are unaltered. Some of the PA$^+$ transcipients were found to have

inherited plasmids which were apparently stable cointegrates of pXO1 and pXO12. These strains, in addition to being able to mobilize pBC16, were shown to transfer the PA^+ penotype to approximately 50% of the Tc^r transcipients.

In contrast to the transfer of pXO1 and pXO2, mobilization of pBC16 by pXO12 appears to occur by donation. Mobilization by donation has been determined not to require physical contact between conjugative plasmids and those being transferred. Again, evidence for the donation of pBC16 by pXO12 is two-fold. The frequency of transcipients which inherit pBC16 is generally quite high, and restriction analysis of pBC16 after being transferred showed no alteration as compared to pBC16 before transfer. Furthermore Southern hybridization experiments indicates that there is no homology between pXO12 and pBC16.

V. Studies on transformation of B. anthracis, B. cereus, and B. thuringiensis

In recent years a number of reports have appeared in the literature on methods for transfsorming B. thuringiensis. We have tried unsuccessfully to transform B. cereus, B. anthracis, and B. thuringiensis by most, if not all, of the methods that have been reported. This has been the experience with most laboratories that have tried to use the tranformation methods that have been reported. In fact, in some instances, the authors of the papers describing the methods have been unable to repeat their results.

Heierson, et al. (15) recently reported a new method for transforming B. thuringiensis, and we have had some success in adapting their procedure to B. cereus. The procedure is given below.

Transformation procedure for B. cereus. Spores (about 10^7) of B. cereus 569 UM20-1 Str^r Trp^- were inoculated into 10 ml of MinIC broth (no glucose) supplemented with 2% (v/v) L broth in a 20-mm cotton-plugged tube and incubated at 37°C on a shaker (210 rev/min). After 14-15 hours 0.3 ml was transferred to 10 ml of fresh broth and incubation was continued 5.5 hours, at which time there were about 7×10^7 cfu/ml). The culture was transferred to a 16-mm screw cap tube and centrifuged at 3700 rev/min for 10 min at 21°C. The cells were washed with 5 ml of 50 mM Tris hydrochloride (pH 7.5), pelleted again, and resuspended in 7 ml of 50 mM Tris hydrochloride-30% sucrose (pH 8.1). Five ml was transferred to a 20-mm cotton-plugged tube and incubated 5

min at 37°C on a slow shaker (140 rev/min). Following this incubation the cells were centrifuged at 3700 (rev/min) for 10 min at 21°C and the cell pellet was resuspended in 0.5 ml of LBP (1:1 mixture of 2X L broth and 0.2 M sodium-potassium phosphate, pH 6.4). (The sodium-potassium phosphate solution contained 1.905 g of KH_2PO_4 and 0.852 g of Na_2HPO_4 per 100 ml. No adjustment of pH was necessary.). DNA (50 µl, about 5 µg) extracted from cells by our modified Kado and Liu procedure was added, and this was followed by addition of 1.5 ml of 40% PEG (w/v, prepared in sodium-potassium phosphate buffer) and the suspension was mixed. Following incubation at 37°C for 15 min on a slow shaker (140 rev/min), the suspension was centrifuged at 3700 rev/min for 10 min at 21°C and the pellet was resuspended in 1 ml of LG broth (L broth with 0.1% glucose added asceptically). Samples (0.1 ml) of the resuspended cells were spread on L agar plates which were then incubated at 37°C for 2-2.5 hours. After this period of time to allow phenotypic expression of plasmid determinants, 2.5 ml of soft L agar (0.5% agar) supplemented with the appropriate antibiotic was poured over each plate and allowed to harden. Incubation of these plates was then continued at 37°C for about 24 hours. Transformants were streaked to selective plates to confirm resistance to the antibiotic and retention of their original auxotrophic marker.

Results. The 2.8-Mdal tetracycline-resistance plasmid pBC16 and the 3.0-Mdal kanamycin-resistance plasmid pUB110 have been transformed into B. cereus 569 by this procedure on several occasions, with pBC16 yielding 90-130 transformants per plate and pUB110 yielding 70-90 transformants per plate.

Some variations of the procedure have been tested and the following observations have been made: (1) Addition of 2% L broth to the Min 1C broth appears to enhance growth of the cells and is necessary for the cells to attain their "competent" state; (2) The pH of the Tris-sucrose buffer is important. No transformants were obtained when buffers of pH 8.4, 8.9 and 9.0 were used; (3) At the time of DNA addition microscopic examination of the suspension showed the presence of cells only; no protoplasts could be seen; (4) This procedure worked better with B. cereus 569 than with B. thuringiensis. With B. thuringiensis subsp. galleria no more than 5-10 Tc^r transformants per plate were obtained.

We have not yet tried this procedure for transforming B. anthracis. However, we will be doing so in the near future. Although the frequency of transformation of B. cereus by this method is quite low, we are optimistic

that we will be able to improve the frequency and also that we can adapt the procedure to B. anthracis. We have tried many procedures in the past and this is the only one that has given positive results.

TABLE 1. List of bacterial strains

Strain	Characteristics and/or source
B. anthracis	
4229 (Pasteur)	Cap^{+c} Tox^{-} (pXO2) [B. Ivins]
4229 R1	pXO2^{-}, Spontaneous Cap^{-} of 4229
4229 UM12	Nal^{r} by UV^{a} of 4229, (pXO2)
4229 tr299-3	pXO2, pXO12, pBC16
PM-36	Vollum strain, (pXO1, pXO2) [MRE^{b}]
PM-36 R1	Cap^{-} spontaneous pXO1^{-} from PM-36, (pXO1)
ΔAmes-1	Cap^{+c} Tox^{-} pXO1^{-} (pXO2) [B. Ivins]
Ames ANR-1	Cap^{-} Tox^{+} pXO2^{-} (pXO1) [B. Ivins]
New Hampshire ΔNH-1	Cap^{+c} Tox^{-} pXO1^{-} (pXO2) [B. Ivins]
New Hampshire NNR-1	Cap^{-} Tox^{+} pXO2^{-} (pXO1) [B. Ivins]
New Hampshire NNR-1Δ1	pXO1^{-} from NNR-1
M	Cap^{+a} Tox^{+} (pXO1, pXO2.1)
M UM2	UM1 cured of pXO1 by growth at 42°, Str^{r} (pXO2.1)
M UM6	pXO2.1^{-}, Spontaneous Cap^{-} from M
Weybridge	Cap^{-} Tox^{+} (pXO1) pXO2^{-} [MRE]
UM44	Ind^{-} by UV of Weybridge, (pXO1)
UM44-1	*str-1* by UV of UM44, (pXO1)
UM44-1 C9	pXO1^{-} of UM44-1 at 42°
UM44-1 C9 tr156G-2	(pXO1-pXO12^{d}, pBC16)
UM44-1 C9 tr156G-2 H1	(pXO1-pXO12); pBC16^{-} at 42°
UM44-1 C9 tr156G-5	(pXO1:pXO12, pBC16)
UM44-1 C9 tr156G-6	(pXO1:pXO12, pBC16)

TABLE 1. (continued)

UM44-1 tr203-23	Tc^r Cry^+ (pXO1, pXO12, pBC16)
UM44-1 tr203-23 C1-4	Tet^s Cry^+ $pBC16^-$ (pXO1, pXO12Δ203)
UM44-1 tr203-23 C1-4 td1	Tc^r by td of tr203-23 C1-4, Cry^+ Tra^- (pXO1, pXO12Δ203, pBC16)
UM44-1 tr203-23 C1-413	Ind^- Str^r Tet^s Cry^+ $pBC16^-$ (pXO12Δ203)
Weybridge A	Colonial variant of Weybridge, (pXO1)
A UM2	Ind^- by UV of Weybridge A, (pXO1)
A UM2 tr244-1	Ind^- Tc^r Cry^+ (pXO1.1, pXO12, pBC16)
A UM2 tr244-1 CN3 H2	(pXO1.1); $pXO12^-$ with novobiocin; $pBC16^-$ at 42^o
A UM17	Ade^- by UV of Weybridge A, (pXO1)
A UM17 tr57B-6	Ade^- Tc^r Cry^+ (pXO1, pXO12, pBC16)
A UM18	pyrA by UV of Weybridge A, (pXO1)
A UM18 td2	UM18(pBC16) by td
A UM18 td2 C25	UM18 td2 $pXO1^-$ $pBC16^-$ by growth at 42^oC
A UM18 td2 C25-1	Spontaneous Str^r of td2-C25
A UM18 td2 C25-1 tr43G-11	Tc^r Cry^+ Str^r (pXO1, pXO12, pBC16)
A UM18 td2 C25-1 tr43G-11 CN1	Cured of pXO12 with novobiocin, (pXO1, pBC16)
A UM18 td2 C25-1 tr43G-11 CH2	$pXO1^-$ of tr43G-11 by growth at 42^o, Cry^+ Tra^- (pXO12Δ43, pBC16)
A M18 td2 C25-1 tr43G-11 C5	$pBC16^-$ of tr43G-11 CH2 by gr wth at 42^o, Cry^+ (pXO12Δ43
A UM23	Ura^- by UV of Weybridge A (pXO1)
A UM23 C1	Ura^-, $pXO1^-$ of A UM23
A UM23 C1-1	str-2 by UV of A UM23 C1
A UM23 C1-1 td20G	pXO2 from tr299-3 by td of A UM23 C1-1
A UM23 C1-1 td22G	pXO2 from tr305-5 by td of A UM23 C1-1
A UM23 C1-1 td25G	pXO2 from tr305-1 by td of A UM23 C1-1

TABLE 1. (continued)

A UM23 C1-1 tr47G-34	Ura$^-$ Tc^r Cry$^+$ Tox$^+$ (pXO1.2, pXO12, pBC16)
A UM23 C1-1 tr47G-34 CN2	(pXO1.2), pBC16$^-$ pXO12$^-$ of tr47G-34 with novobiocin
A UM23 C1-1 tr338A-1	Ura$^-$ Tc^r Tra$^+$ (pXO14, pBC16)
A UM23 C1-1 tr338A-1 C1	pBC16$^-$ of tr338A-1
A UM23 C1-1 tr359A-1	Ura$^-$ Tc^r (pXO13, pBC16)
A UM23C1-1 tr359A-1 C1	Ura$^-$ Str^r Tet^s Tra$^+$ (pXO13) pBC16$^-$
A UM23 C1-2	A UM23 C1 rfm-1 by td from A UM693 His$^-$ rfm-1
A UM23 C1-2 tr135K-1	Ura$^-$ Rif^r Em^r Tra$^+$ (pXO503)
A UM23 C1-2 tr201G-1	(pXO1.3, pBC16)
A UM23 C1-2 tr201G-1 H3	(pXO1.3), pBC16$^-$ at 42^o
A UM23 C1-2 tr201G-2	(pXO1, pXO12, pBC16)
A UM23 C1-2 tr220G-2	(pXO1:pXO12^c, pBC16)
A UM23 C1-2 tr220G-3	(pXO1, pXO12, pBC16)
A UM23 C1-2 tr220G-4	(pXO1:pXO12, pBC16)
A UM23 C1-2 tr272G-2	(pBC16)
A UM23 C1-2 tr689B-3	Em^r Tc^r Cry$^+$ Tra$^+$ (pXO12, pXO503, pBC16)
A UM23 C1-2 tr706B-5	Em^r Cry$^-$ Tra$^+$ carries pXO12-706 which is pXO12::Tn917
A UM23 C2	pXO1$^-$ of A UM23, Ura$^-$
A UM23 C2 tr96B-3	Ura$^-$ Tc^r (pXO11, pBC16)
A UM23 C2 tr96B-3-C19	pBC16$^-$ of tr96B-3, Ura$^-$ Tet^s (pXO11)
A UM23 C2 tr237-10	Ura$^-$ Tc^r Cry$^+$ (pXO12, pBC16)
A UM121	Ura$^-$ Rif^r Tc^r (pXO503); 3335 UM8 x A UM23 C1-2
B. cereus	
569	wild type (pXO3, pXO4, pXO5) [NRRL^e]
569K	Trp$^-$ Str^r [A. Aronson]

TABLE 1. (continued)

569 L21	569 lysogenized with TP-21
569 UM20	Ant$^-$ by UV of 569 (pXO3)
569 UM20-1	Str^r by UV of UM20 (pXO3)
569 UM20-1 tr251-1	Ant$^-$ Str^r Tc^r Cry$^+$ (pXO3, pXO12, pBC16)
569 UM20-1 tr305-1	str-1 (pXO2, pXO3, pXO12, pBC16)
569 UM20-1 tr305-5	str-1 (pXO2, pXO3, pXO12, pBC16)
569 UM20-1 tr374B-5	Ant$^-$ Str^r Tc^r Cry$^+$ Tra$^+$ (pXO12, pBC16)
569 UM20-1 tr374B-5 C1	Tet^s pBC16$^-$ of tr374B-5 by growth at 42°
569 UM20-1 tr691B-12	(pLS20::T n917, pXO12, pBC16)
569 UM20-1 tr730B-14	Em^r Cry$^-$ Tra$^+$ (pXO12-730 which is pXO12::Tn917)
569 UM25-9	met-4 trp-3 str-9
569 UM27-11	met-4 ile-3 str-11
T	wild type (terminalis)
B. thuringiensis	
1328	wild type, subspecies not known [R. E. Gordon]
subsp. kurstaki HD1-9	carries TP-21 [A. Aronson]
subsp. kurstaki HD1-9 td1	(pBC16) by td of HD1-9
4042A	subsp. thuringiensis, Cry$^+$ (pXO11, pXO12)
4042A UM2-1	pyrA Str^r (pXO11, pXO12)
4042A UM15	Trp$^-$ by UV of 4042A
4042B	subsp. aizawai [NRRL]
B. megaterium	
PV229	Leu$^-$ Str^r Pep$^-$ Meg$^-$ [P. Vary]
B. subtilis	
168	trpC2 [M. Fox]

TABLE 1. (continued)

168 UM21	Leu$^-$ Met$^-$ Str^r
168 UM42	Leu$^-$ Met$^-$ Str^r MLS^r (pXO503); IG-20 UM6 x^g 168 UM21
PSL1	r$^-$m$^-$ recE Arg$^-$ Thr$^-$ Leu$^-$, BGSC^f strain 1A510
PSL1 UM1	r$^-$m$^-$ Arg$^-$ Thr$^-$ Leu$^-$ Spontaneous Str^r from PSL1
PSL1 UM3	r$^-$m$^-$ Arg$^-$ Thr$^-$ Leu$^-$ Str^r Tc^r Cm^r MLS^r (pLS20, pBC16, pTV1) by tf of PSL1 UM13
PSL1 UM4	PSL1 UM12 pBC16$^-$ by curing
PSL1 UM11	PSL1 UM4(pUB110) by tf
PSL1 UM12	r$^-$m$^-$ Arg$^-$ Thr$^-$ Leu$^-$ Str^r Tc^r (pLS20, pBC16); 3335 UM8 x PSL1 UM1
PSL1 UM13	r$^-$m$^-$ Arg$^-$ Thr$^-$ Leu$^-$ Str^r Tc^r (pLS20, pBC16); 3335 UM8 x PSL1 UM1
IG-20	r$^-$m$^-$ Trp$^-$; BGSC strain 1A436
IG-20 UM1	r$^-$m$^-$ Trp$^-$ Rif^r; UV of IG-20
IG-20 UM2	r$^-$m$^-$ Trp$^-$ Str^r; Spontaneous from IG-20
IG-20 UM3	r$^-$m$^-$ Trp$^-$ Rif^r Tc^r (pLS20, pBC16); PSL1 UM3 x IG-20 UM1
IG-20 UM4	r$^-$m$^-$ Trp$^-$ Rif^r MLS^r (pXO501); PSL1 UM3 x IG-20 UM1
IG-20 UM5	r$^-$m$^-$ Trp$^-$ Rif^r MLS^r (pXO502); PSL1 UM3 x IG-20 UM1
IG-20 UM6	r$^-$m$^-$ Trp$^-$ Rif^r MLS^r (pXO503, pBC16); PSL1 UM3 x IG-20 UM1
IG-20 UM7	r$^-$m$^-$ Trp$^-$ Rif^r MLS^r (pXO504, pBC16); PSL1 UM3 x IG-20 UM1
PY143	Cm^r MLS^r (pTV1) [P. Youngman]
W23	Str^r [M. Fox]
B. subtilis (natto)	
3335	Pga$^+$ Bio$^-$ (pLS19, pLS20) [T. Hara]

TABLE 1. (continued)

3335 UM2	Rif^r by UV of 3335
3335 UM4	Spontaneous Pga^- $pLS19^-$ from 3335, (pLS20)
3335 UM5	Rif^r by UV of UM4 (pLS20)
3335 UM8	Tc^r by tf of UM4, (pLS20, pBC16)
3335 UM44 tr537B-16	Pga^- Tc^r $pLS20^-$ (pBC16)
3335 UM22	UM4 tr537B-16 $pBC16^-$ at 42^o, no plasmids
3335 UM23	Ade^- of UM22 by UV, no plasmids
3335 UM24	Str^r of UM23 by UV, no plasmids
3335 UM25	rfm-7 of UM22 by UV, no plasmids
3335 UM27	Bio^- Ade^- Str^r Tc^r (pLS20 pBC16), 3335 UM8 x 3335 $UM24^c$
3335 UM28	Bio^- Ade^- Str^r Tc^r (pBC16), 3335 UM8 x 3335 UM24
3335 UM31	Bio^- Rif^r Tc^r (pLS19, pLS20, pBC16), 3335 UM27 x 3335 UM2
B. licheniformis	
9945A	Prototrophic, Pga^+ [C. Thorne]
9945A UM89	Lys^- Str^r
B. pumilus	
12140 L9S1	Lys^- [P. Lovett]
12140 L9S1 UM1	Lys^- Str^r; UV of 12140 L9S1
Escherichia coli	
JM83	(pHT44) [M.-M. Lecadet]

Abbreviations: Ade, adenine; Ant, anthranilic acid; Arg, arginine; Bio, biotin; Ura, uracil; Ind, indole; C, cured; Cm^r, pTV1-encoded chloramphenicol resistance; Cap, synthesis of capsule; Cap^{+a}, synthesis of capsules in air; Cap^{+c}, CO_2-dependent capsule synthesis; Cry, synthesis of parasporal crystals; Em^r, erythromycin resistant; Leu, leucine; Lys, lysine; Met, methionine; Thr,

TABLE 1. (continued)

threonine; Trp, tryptophane; MLS^r, Tn917-encoded macrolide, lincosamide, and streptogramin B resistance, Nm^r, pUB110-encoded neomycin resistance; N, curing by treatment with novobiocin; Pga, synthesis of polyglutamic acid; pyrA, mutation conferring requirement for arginine plus uracil; Rif^r, phenotype for rifampicin resistance; rfm, genotype for rifampicin resistance; Str^r, streptomycin resistant; Tc^r, pBC16-encoded tetracycline resistance; Tet, tetracycline; td, transduction; tf, transformation; Tox, synthesis of toxin; Tra, mediation of plasmid transfer by mating; Ura, uracil. In strain designations, tr denotes a transcipient strain and td denotes a transductant obtained by CP-51-mediated transduction. Names or abbreviations in brackets represent sources of particular strains.

[a]UV, Mutagenesis by UV light.

[b]MRE, Microbiological Research Establishment, Porton, England.

[c]pXO1:pXO12 denotes a presumed cointegrate plasmid of pXO1 and pXO12.

[d] pXO1-pXO12 denotes a recombinant plasmid of pXO1 and pXO12.

[e]NRRL, Northern Regional Research Laboratory, Department of Agriculture, Peoria, Illinois.

[f] Bacillus Genetics Stock Center, Columbus, Ohio.

[g] x is preceded by the donor strain and followed by the recipient strain, and indicates that the isolate was derived from a mating.

TABLE 2. Test of some Bacillus species as recipients of pBC16 in matings with B. subtilis (natto) 3335 UM8 (pLS20, pBC16)

Recipient strain	Tc^r transcipients[a] No. per ml	Frequency[b]
B. anthracis UM44-1C9	1.3×10^3	1.3×10^{-5}
B. cereus UM20-1	3.4×10^3	7.1×10^{-4}
B. thuringiensis 4042A UM2-1	1.6×10^3	3.6×10^{-4}
B. licheniformis 9945A UM89	1.4×10^4	2.6×10^{-3}
B. megaterium PV229	3.5×10^1	2.3×10^{-6}
B. pumilus 12140 L9S1 UM1	1.4×10^4	2.8×10^{-3}
B. subtilis PSLI UM1	2.8×10^2	2.5×10^{-5}
B. subtilis 168 UM21	9.5×10^4	2.7×10^{-3}
B. subtilis W23	3.8×10^2	2.7×10^{-2}
B. subtilis (natto) 3335 UM24	1.7×10^4	7.3×10^{-4}

[a] Data represent the average results of three experiments.

[b] Frequency is expressed as the number of transcipients per donor.

TABLE 3. Cotransfer of pLS20 and pBC16

Donor strain	Recipient strain	Frequency of Tc^r transcipients	% Cotransfer of pLS20[a]
B. subtilis (natto) 3335 UM8	B. subtilis (natto) 3335 UM24	1.1×10^{-3}	43.8 (7/16)
B. subtilis (natto) 3335 UM8	B. subtilis 168 UM21	3.8×10^{-4}	0 (0/82)
B. subtilis (natto) 3335 UM8	B. subtilis IG-20 UM2	6.2×10^{-5}	0 (0/168)
B. subtilis (natto) 3335 UM8	B. subtilis PSLI UM1	2.5×10^{-5}	28.6 (4/14)
B. subtilis PSLI UM3	B. subtilis IG-20 UM1	9.0×10^{-3}	1.5 (3/196)
B. subtilis IG-20 UM3	B. subtilis 168 UM21	4.4×10^{-3}	30.5 (46/151)

[a] Percent of $pBC16^+$ transcipients which also acquired pLS20, as indicated by results of plasmid extractions and/or replica plate matings. Numbers in parentheses represent the number of Tc^r transcipients which acquired pLS20 over the number of Tc^r transcipients tested.

TABLE 4. Effectiveness of *B. subtilis* PSL1 UM12(pLS20, pBC16) and *B. subtilis* PSLI UM11(pLS20, pUB110) as donors of pBC16 or pUB110

Recipient strain	Tc^r transcipients[a]		Nm^r transcipients[b]	
	No. per ml	Frequency	No. per ml	Frequency
B. anthracis UM23C1-2	1.8×10^3	1.6×10^{-4}	6.5×10^3	2.3×10^{-4}
B. subtilis IG-20 UM1	3.8×10^3	4.5×10^{-4}	3.0×10^3	1.3×10^{-4}
B. subtilis (natto) 3335 UM25	7.3×10^3	4.9×10^{-4}	1.4×10^3	3.8×10^{-5}

[a] All of 16 Tc^r transcipients tested from each mating acquired pBC16.

[b] All of 8 Nm^r transcipients tested from each mating acquired pUB110.

TABLE 5. Test of pLS20::Tn*917*$^{+}$ primary transcipients as donors in matings with *B. subtilis* 168 UM21

Donor strain	MLS^{r} transcipients			Tc^{r} transcipients		
	No. per ml	Frequency	% Cotransfer[a]	No. per ml	Frequency	% Cotransfer[b]
B. subtilis IG-20 UM4 (pXO501)	2.0×10^{2}	1.3×10^{-6}				
B. subtilis IG-20 UM5 (pXO502)	1.0×10^{3}	1.3×10^{-5}				
B. subtilis IG-20 UM6 (pXO503, pBC16)	8.0×10^{2}	5.0×10^{-5}	71.4 (10/14)	1.6×10^{3}	1.1×10^{-4}	60.6 (20/33)
B. subtilis IG-20 UM7 (pXO504, pBC16)	1.0×10^{3}	6.7×10^{-4}	71.4 (10/14)	8.5×10^{3}	2.4×10^{-3}	30.0 (36/120)
B. subtilis IG-20 UM3 (pLS20, pBC16)				6.6×10^{4}	4.4×10^{-3}	30.5 (46/151)

[a] Percent of pLS20::Tn*917*$^{+}$ transcipients which also acquired pBC16, as indicated by results of plasmid extractions. Numbers in parentheses represent the number of pBC16^{+} transcipients over the number of MLS^{r} transcipients tested.

[b] Percent of pBC16^{+} transcipients which also acquired pLS20::Tn*917* or pLS20, as indicated by results of plasmid extractions and/or tests for MLS^{r}. Numbers in parentheses represent the number of pLS20::Tn*917*$^{+}$ or pLS20^{+} transcipients over the number of Tc^{r} transcipients tested.

TABLE 6. Complement of EcoRI restriction fragments of pLS20 and pX0503

Fragment no.	Fragment size (kb)[a]	
	pLS20	pX0503
1A		9.25
1	7.25	7.25
2	6.4	6.4
3	5.9	5.9
4	5.0	5.0
5	4.1	
6	3.35	3.35
7	3.1	3.1
8	3.05	3.05
9	2.75	2.75
10	2.5	2.5
11	1.9	1.9
12	1.8	1.8
13	1.65	1.65
14	1.6	1.6
15	1.55	1.55
16	1.45	1.45
17	1.3	1.3
Sum:	54.65	59.8

[a] Data represent the average results of three experiments.

TABLE 7. Test for requirement of cell-to-cell contact for transfer of chromosomal markers from *B. thuringiensis* to *B. cereus*

Recipient	*B. thuringiensis* subsp. *kurstaki* HD1-9 donor	
	Mating condition	No. of prototrophic transcipients per ml
B. cereus		
569 UM25-9	mixed	2550
569 UM25-9	U-tube	960
569K	mixed	1410
569K	U-tube	100

U-tube matings contained 3 ml each of donor and recipient cultures separated by a 0.45 µm Millipore membrane. Mixed broth matings contained 0.5 ml each of donor and recipient cultures together in 20-mm tubes. Prototrophic transcipients were selected on Minimal 4NH agar plates containing streptomycin (1 mg/ml).

TABLE 8. Phage titers from induced cultures of HD1-9 and HD1-9 td1(pBC16)

Strain	Growth medium	Method of induction	Titer
B. thuringiensis			
HD1-9	L broth	UV light	8.0×10^7
	L broth	Mitomycin C	4.5×10^8
	L broth	Spontaneous	3.0×10^7
	BHI broth	Spontaneous	1.4×10^9
HD1-9 td-1	L broth	UV light	4.4×10^8
	L broth	Mitomycin C	4.6×10^8
	BHI broth	Mitomycin C	2.3×10^9

For spontaneously produced phage, cultures were grown overnight and filtered. For induction with mitomycin C, 25 ml of broth was inoculated with 2.5 ml of a 16-h shaken culture and incubated for 2 h at 37°C. Mitomycin C (0.4 µg/ml) was added and incubation was continued for 2 h. For UV induction 5 ml of a 2-h culture grown as above was treated with UV light for 60 sec and transferred to 25 ml of fresh broth. Incubation was continued for 2 h. All cultures were filtered through Millipore HA membranes.

TABLE 9. Effect of phage antiserum on transfer of pBC16 and chromosomal markers in mating mixtures

B. thuringiensis Donor	Addition to mating mixture	*B. cereus* 569K transcipients per ml	
		Prototrophic	Tc^r
kurstaki HD1-9	None	710	0
	TP-12 antiserum	10	0
	Preimmune serum	460	0
kurstaki HD1-9 td-1	None	1110	>10000
	TP-12 antiserum	10	>10000
	Preimmune serum	330	>10000
No donor		10	0

Mating mixtures of HD1-9 and HD1-9 td1(pBC16) donors and *B. cereus* 569K recipients were plated on Minimal 4NH agar with streptomycin (1 mg/ml) to select for prototrophic transcipients and on L agar plus tetracycline (25 µg/ml) and streptomycin (1 mg/ml) to select for tetracycline-resistant transcipients.

TABLE 10. Determination of host range of the specialized transducing phage TP-21::Tn*917*

Strain	Em^r transductants per ml
B. anthracis	
4229 R1(pXO2)$^-$	3.3×10^5
Vollum PM36 R1(pXO1)	2.9×10^2
Ames ANR-1(pXO1)	3.0×10^1
ΔAmes-1(pXO2)	0
M UM6(pXO1)	2.1×10^2
M UM2(pXO2)	1.0×10^1
New Hampshire NNR-1(pXO1)	1.0×10^2
New Hampshire ΔNH-1(pXO2)	7.0×10^1
New Hampshire NNR-1Δ1(pXO1)$^-$	1.1×10^2
Weybridge A UM18(pXO1)	8.0×10^1
Weybridge UM44-1(pXO1)	1.1×10^2
B. cereus	
569	2.4×10^5
569 L21(TP-21)	3.2×10^3
T	0
B. thuringiensis	
subsp. *kurstaki* HD1-9	7.4×10^3
subsp. *thuringiensis* 4042A UM15	2.0×10^1
subsp. *aizawai* 4042B	3.6×10^3

TABLE 11. Transfer frequency of pXO12::Tn917 derivatives

Donor		Recipient	Transcipients	
Strain	Plasmids		No. per ml	Frequency
B. anthracis UM23 C2 tr237-10	pXO12, pBC16	B. anthracis	9.0×10^5	1.9×10^{-2}
		B. cereus	3.9×10^6	7.1×10^{-2}
B. cereus 569 UM20-1 tr251-1	pXO12, pBC16	B. anthracis	4.8×10^4	1.6×10^{-4}
B. anthracis UM23 C1-2 tr706B-5	pXO12::Tn917	B. anthracis	2.3×10^5	1.3×10^{-2}
		B. cereus	4.5×10^5	2.6×10^{-2}
B. cereus 569 UM20-1 tr730B-14	pXO12::Tn917	B. anthracis	2.2×10^4	9.2×10^{-5}

[a]To counterselect the donor, an appropriate antibiotic resistant recipient was employed.

[b]Frequency is expressed as the number of transcipients per donor.

TABLE 12. Transfer of pXO1 from B. anthracis transcipients to cured B. anthracis.

Mating No.	B. anthracis donor	B. anthracis recipient	Tc^r transcipients		
			No. per ml	Frequency	No. PA^+/No. tested
156G	A UM17 tr57B-6 (pXO1, pXO12, pBC16)	UM44-1 C9	1.4×10^3		6/1168 (0.5%)
201G	UM2 tr244-1 (pXO1.1, pXO12, pBC16)	A UM23 C1-2	9.6×10^3	2.2×10^{-4}	2/743 (0.3%)
220G	UM2 tr244-1 (pXO1.1, pXO12, pBC16)	A UM23 C1-2	7.85×10^3	1.1×10^{-4}	5/742 (0.6%)
169G	UM44-1 C9 tr156G-5 (pXO1:pXO12, pBC16)	A UM23 C1-2	3.95×10^5	1.4×10^{-2}	495/874 (57%)
170G	UM44-1 C9 tr156G-6 (pXO1:pXO12, pBC16)	A UM23 C1-2	3.15×10^5	6.4×10^{-3}	432/730 (59%)
229G	A UM23 C1-2 tr220G-2 (pXO1:pXO12), pBC16)	UM44-1 C9	6.75×10^2	2.1×10^{-4}	83/170 (49%)
231G	A UM23 C1-2 tr220G-4 (pXO1:pXO12, pBC16)	UM44-1 C9	5.05×10^2	6.3×10^{-5}	76/170 (45%)

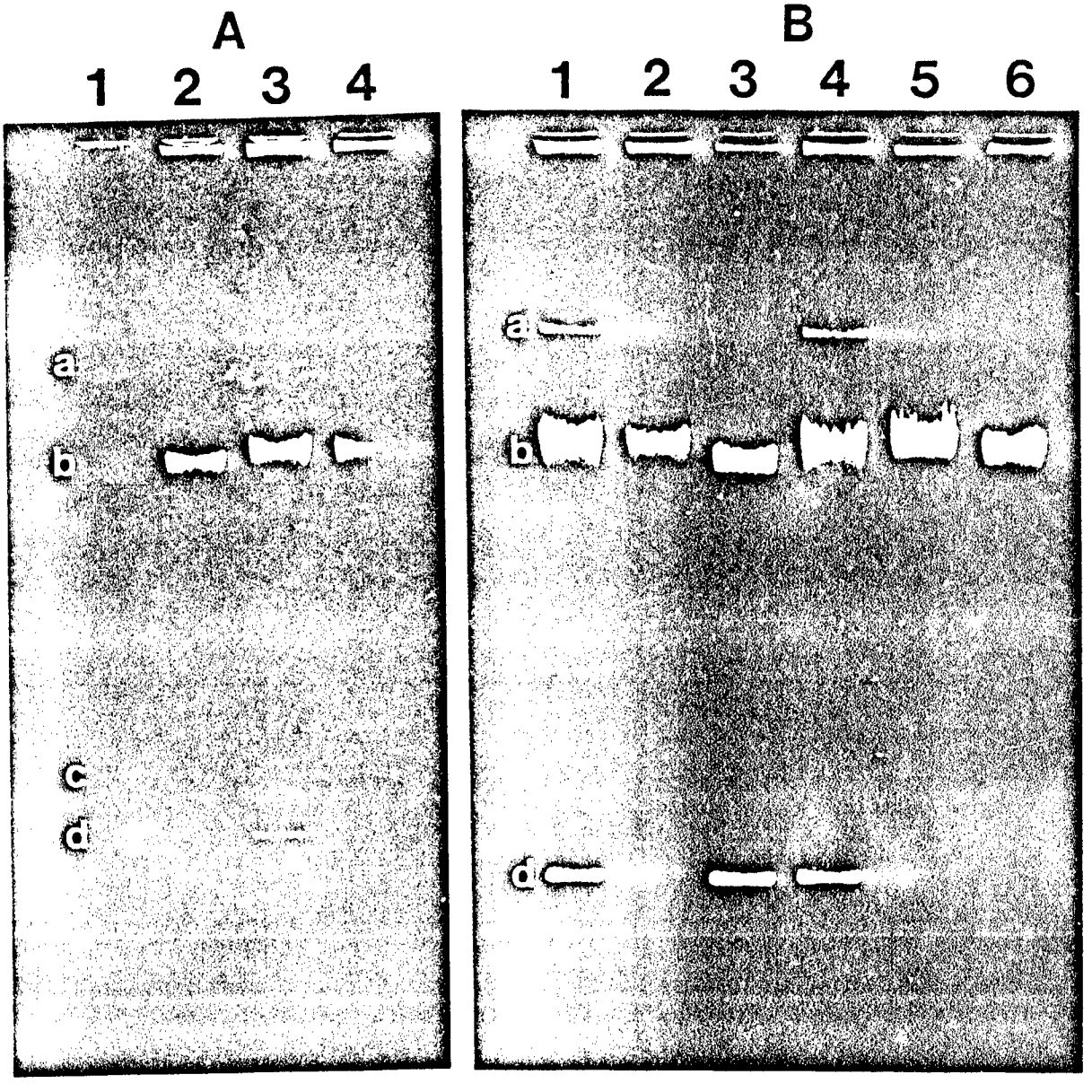


FIG. 1. Agarose gel electrophoresis of plasmid DNA, demonstrating transfer of pBC16 and pLS20. a, pLS20 (55 kb); b, chromosomal DNA; c, pLS19 (5.4 kb); and d, pBC16 (4.2 kb). (A) B. subtilis (natto) 3335 and some derivatives used in this study. Lanes: 1, (natto) 3335; 2, UM4; 3, UM8; 4, UM24. (B) B. subtilis (natto) donor and B. subtilis (natto) and B. anthracis recipients and transcipients. Lanes: 1, B. subtilis (natto) UM8, donor; 2, B. subtilis (natto) UM24, recipient; 3, B. subtilis (natto) UM28, transcipient; 4, B. subtilis (natto) UM27, transcipient; 5, B. anthracis A UM23C1-2, recipient; 6, B. anthracis A UM121, transcipient.

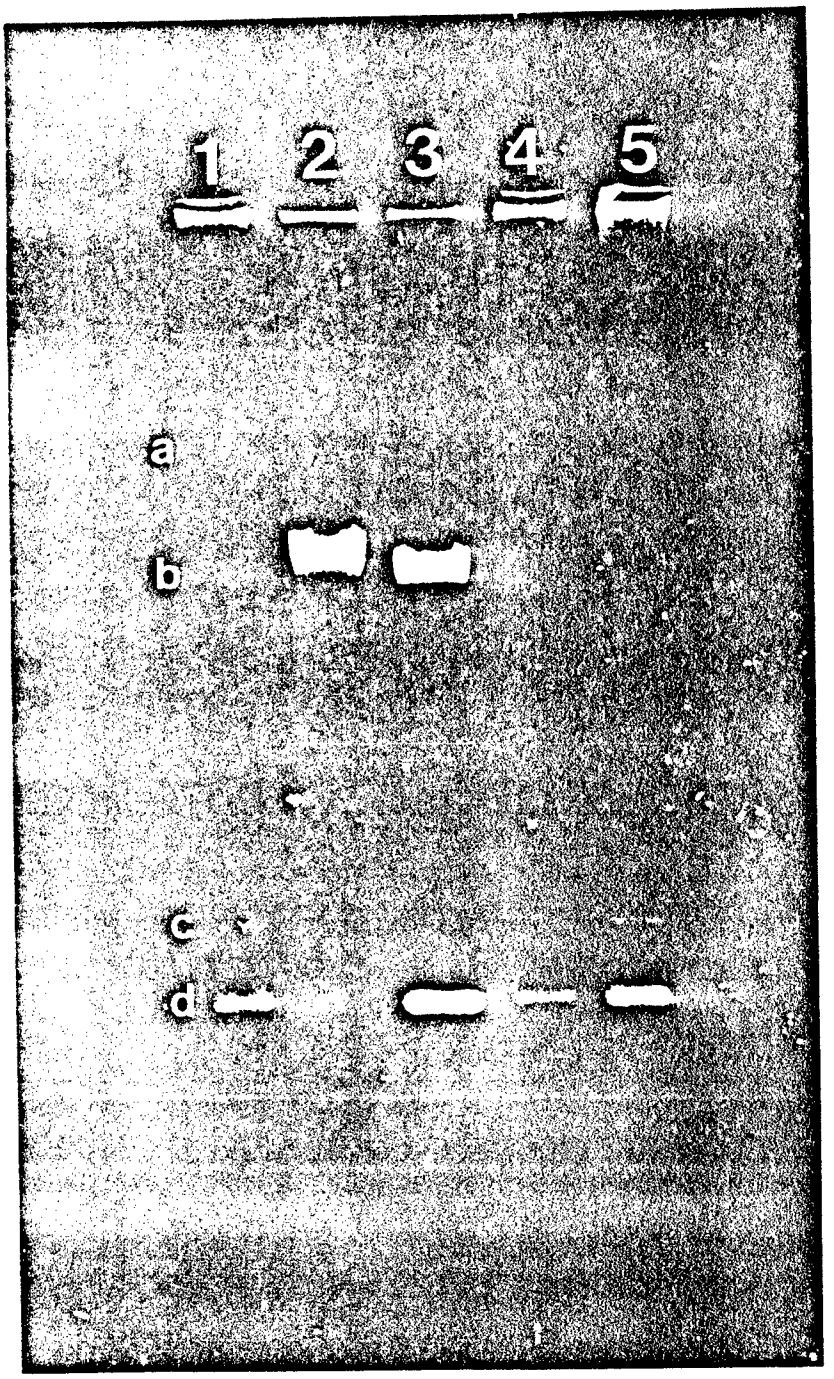


FIG. 2. Agarose gel electrophoresis of plasmid DNA from B. subtilis (natto) strains, demonstrating cotransfer of pLS19 and pBC16. a, pLS20; b, chromosomal DNA; c, pLS19; and d, pBC16. Lanes: 1, UM31, donor; 2, UM24, recipient; 3, UM24 transcipient 1; 4, UM24 transcipient 2; 5, UM24 transcipient 3.

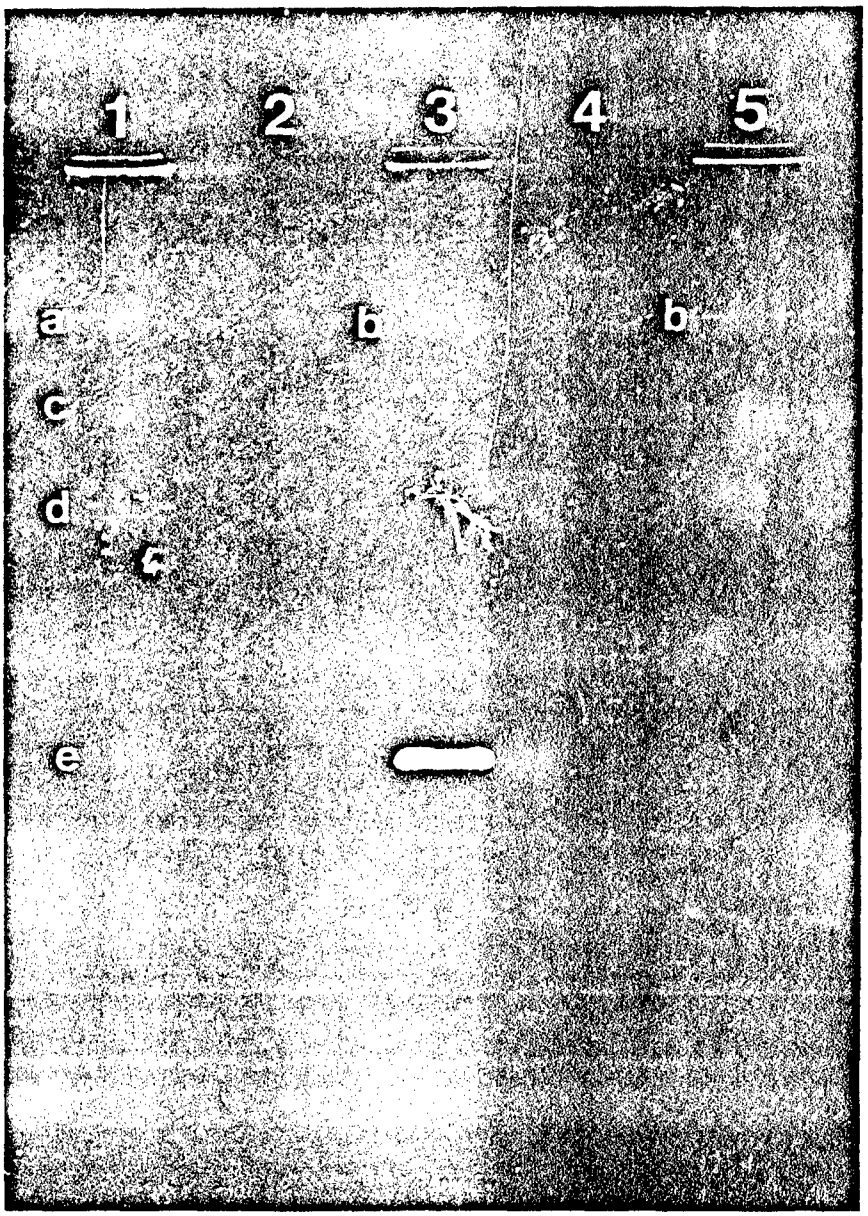


FIG. 3. Agarose gel electrophoresis of plasmid DNA from B. subtilis strains, demonstrating generation and transfer of pXO503. a, pLS20; b, pXO503 (59.8 kb); c, chromosomal DNA; d, pTV1 (12.4 kb); and e, pBC16. Lanes: 1, PSLI UM3, donor; 2, IG-20 UM1, recipient; 3, IG-20 UM6, transcipient; 4, 168 UM21 recipient; 5, 168 UM42, transcipient.

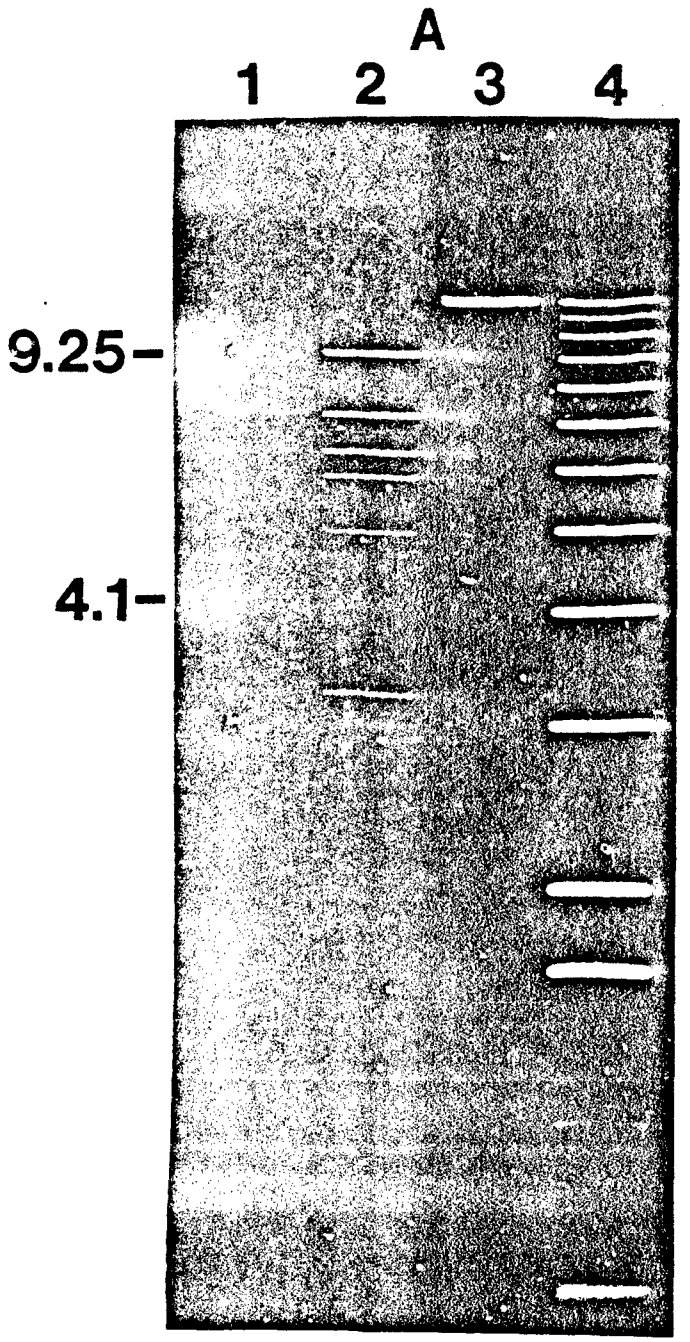


FIG. 4A. Agarose gel electrophoresis of EcoRI-digested plasmid DNA. Fragment sizes are given in kilobases. Lanes: 1, pLS20; 2, pXO503; 3, pTV1; 4, kb ladder.

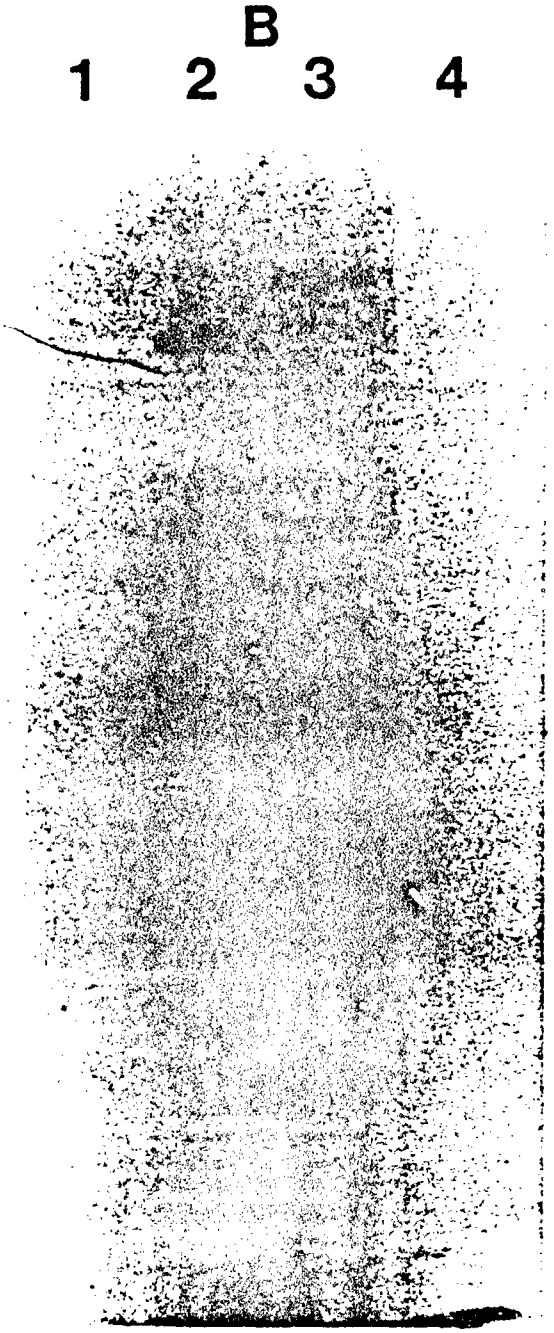


FIG. 4B. Autoradiograph from a Southern blot of EcoRI-digested DNA after hybridization with ^{32}P-labeled pTV1 probe. The lanes correspond to those of the original gel shown in FIG. 4A.

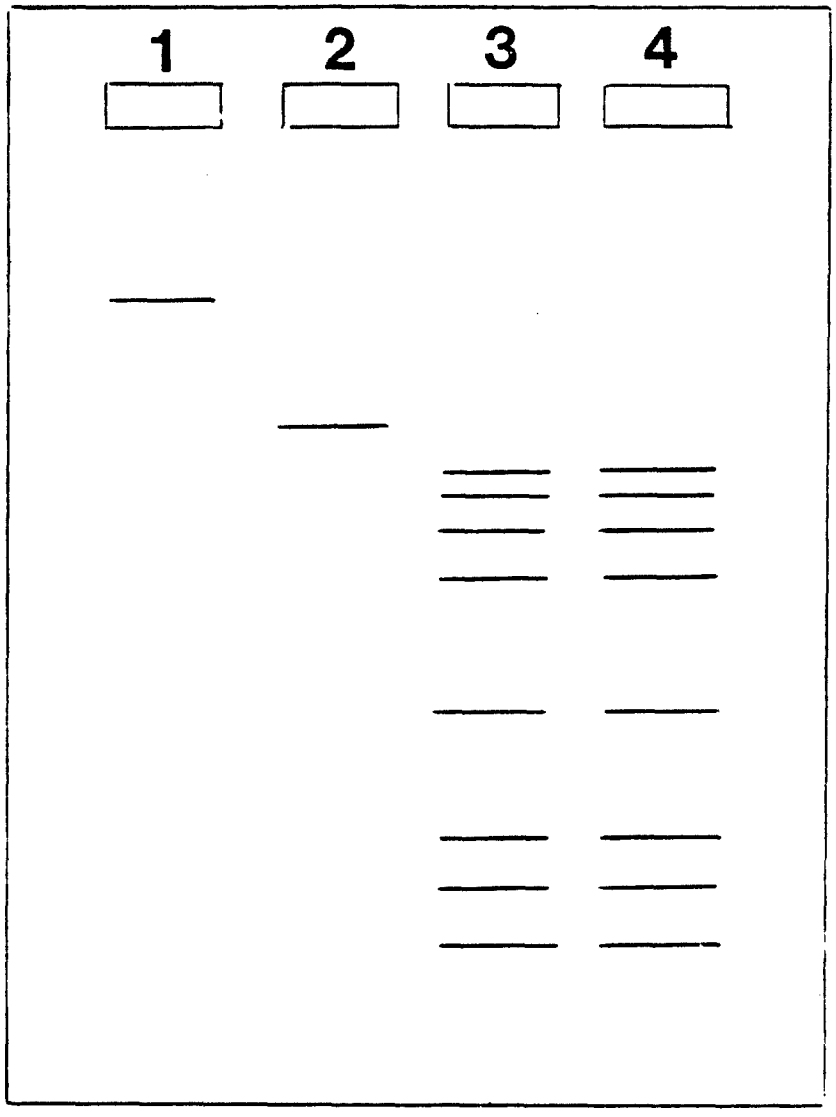


Fig. 5. Schematic of an agarose gel comparing the XbaI restriction banding patterns of the 29-Mdal plasmid from kurstaki HD1-9 and TP-21c phage DNA. Lanes: 1, 29-Mdal plasmid DNA; 2, TP-21c phage DNA; 3, 29-Mdal plasmid DNA cut with XbaI; 4, TP-21c phage DNA cut with XbaI.

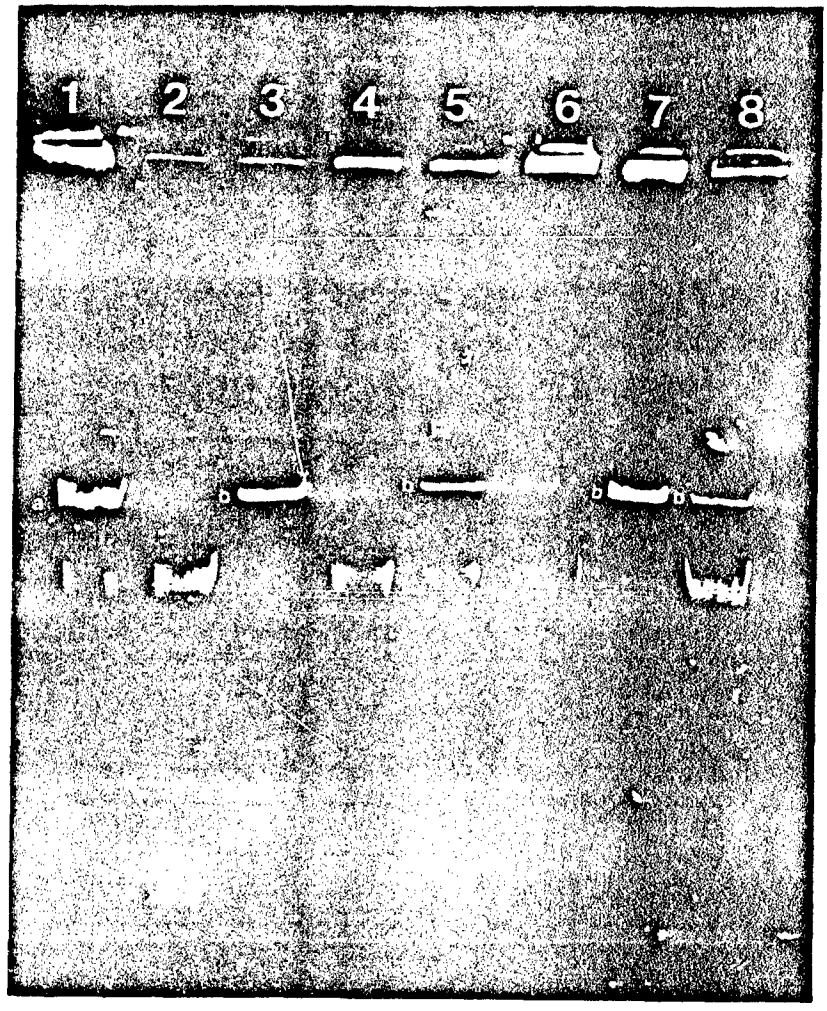


Fig. 6. Agarose gel of plasmid DNA from B. thuringiensis subsp. kurstaki HD1-9 and TP-21::Tn917 lysogens. Plasmid bands: a, 29-Mdal plasmid (TP-21 prophage); b, TP-21::Tn917 . The diffuse band in all lanes is chromosomal DNA. Lanes: 1, B. thuringiensis subsp. kurstaki HD1-9; 2, B. cereus 569; 3, B. cereus 569 (TP-21::Tn917); 4, B. thuringiensis 1328; 5, B. thuringiensis 1328 (TP-21::Tn917); 6, B. anthracis 4229; 7, B. anthracis 4229 (TP-21::Tn917); 8, B. cereus 569 (TP-21::Tn917).

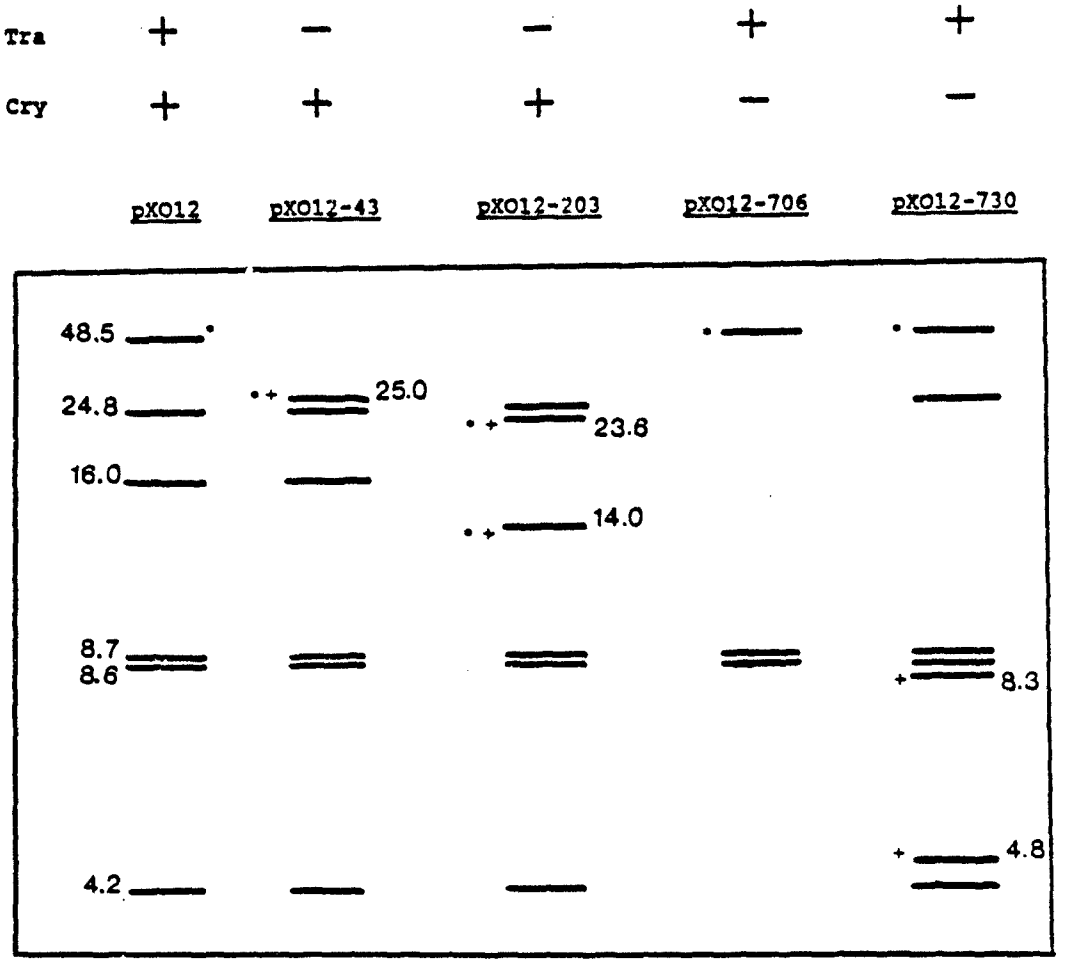
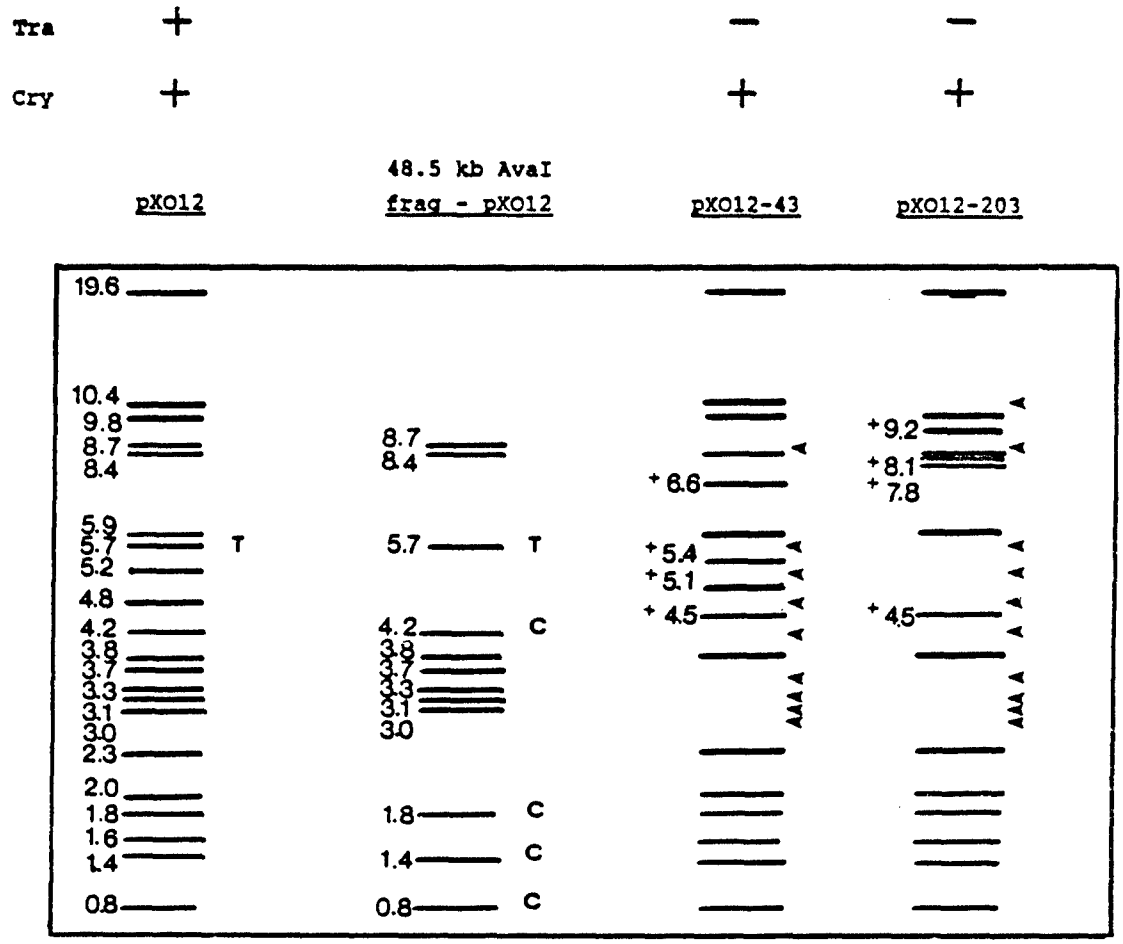


FIG. 7. AvaI restriction pattern of pXO12 plasmid derivataives. The asterisk marks bands that hybridized to the ^{32}P-labeled 48.5-kb fragment of pXO12. Bands marked by "+" were given by the altered plasmids but not by wild-type pXO12. The "+" and "−" signs at the top indicate the phenotype conferred by the plasmids with respect to parasporal crystal synthesis and fertility.



C = BglII fragments contained within the 48.5 kb AvaI fragment of pXO12 that have been cloned in B. subtilis

T = ^{32}p-pHT44 (pUC18::Tn4430)

FIG. 8. BglII restriction pattern of pXO12 plasmid derivatives. Bands marked by "+" were given by the altered plasmids but not by wild-type pXO12. The arrows indicate fragments that were given by wild-type pXO12 but not by the altered plasmids.

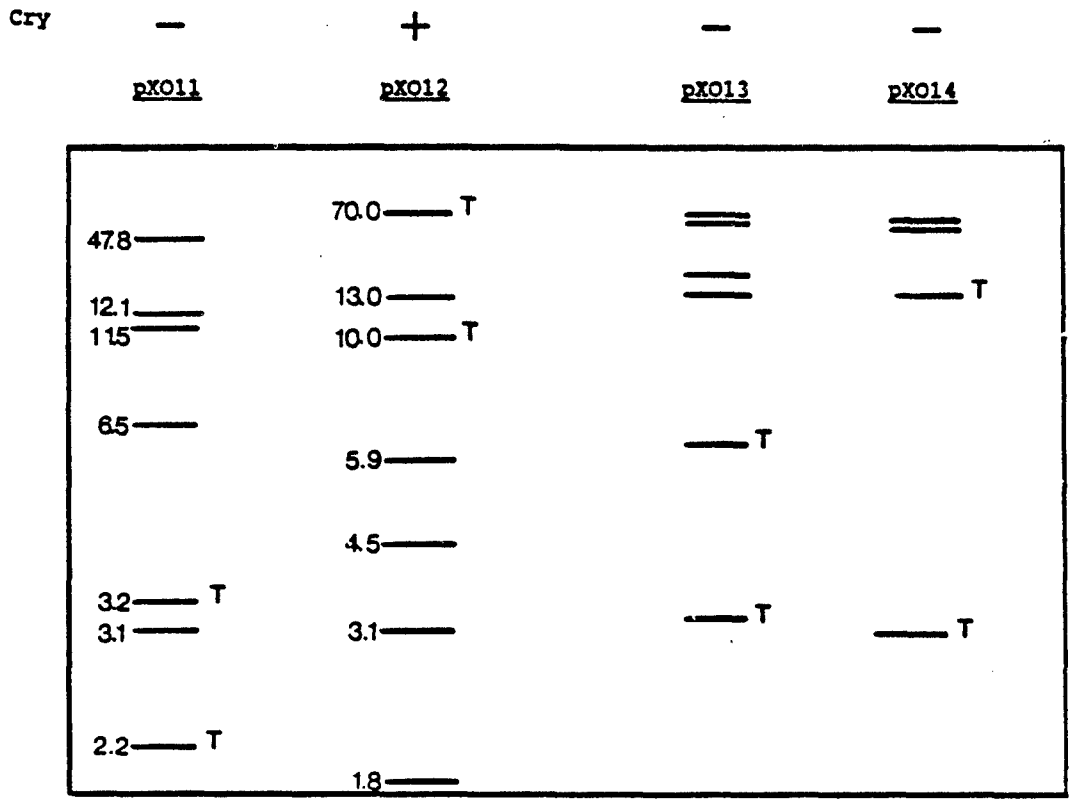


FIG. 9. PstI restriction pattern of B. thuringiensis conjugative plasmids. The fragments marked with "T" hybridized to ^{32}P-labeled pHT44 (pUC18::Tn4430)

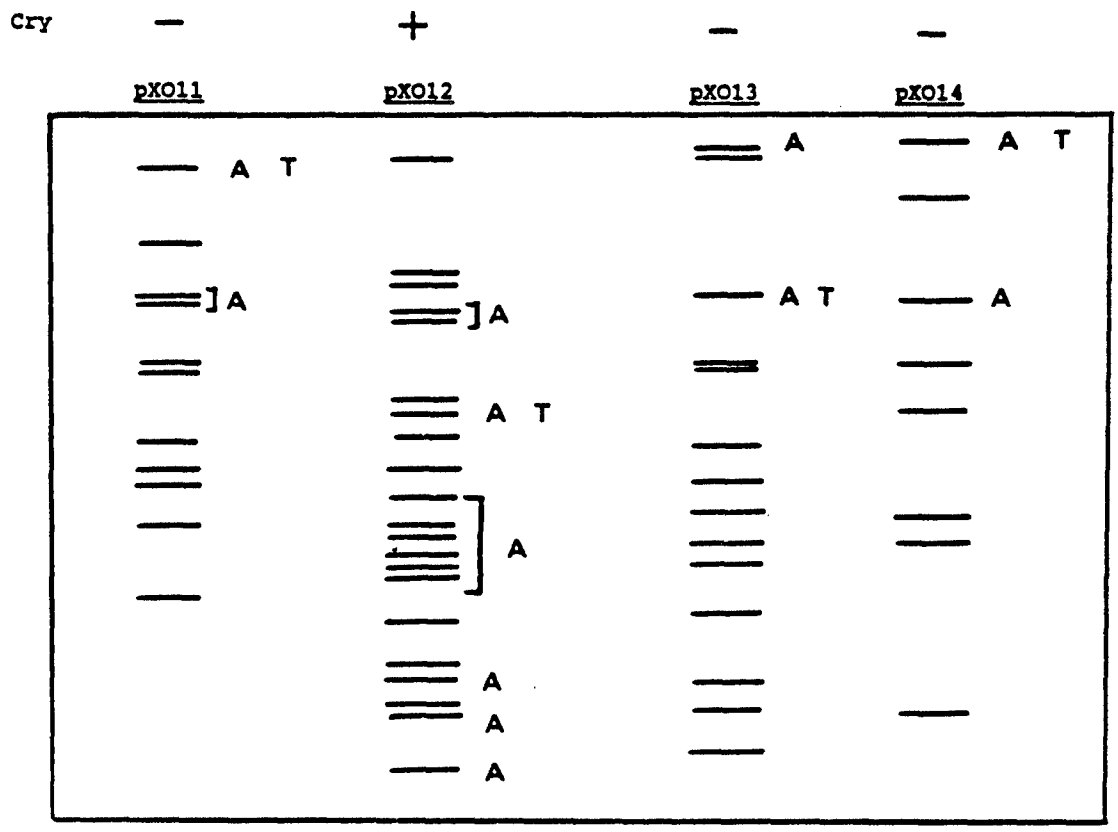


FIG. 10. BglII restriction pattern of B. thuringiensis conjugative plasmids. The fragments marked with "T" hybridized to ^{32}P-labeled pHT44 (pUC18::Tn4430), and those marked with "A" hybridized to ^{32}P-labeled 48.5-kb AvaI fragment from pXO12.

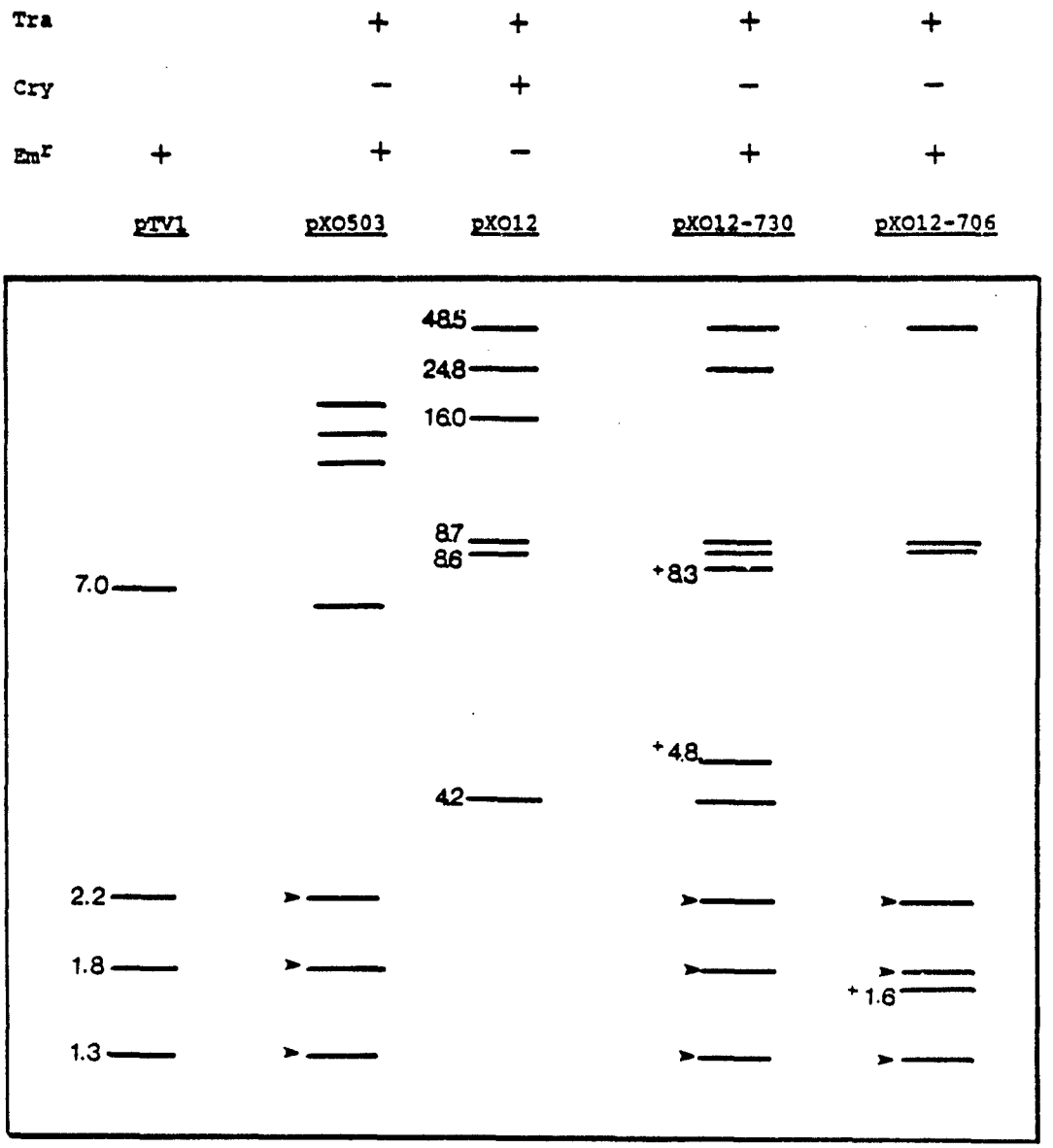


FIG. 11. AvaI restriction pattern of various plasmids, demonstrating the acquisition of Tn917 by pXO12 derivatives pXO12-730 and pXO12-706. The arrows mark fragments that correspond to those given by transposon Tn917 present on pTV1.

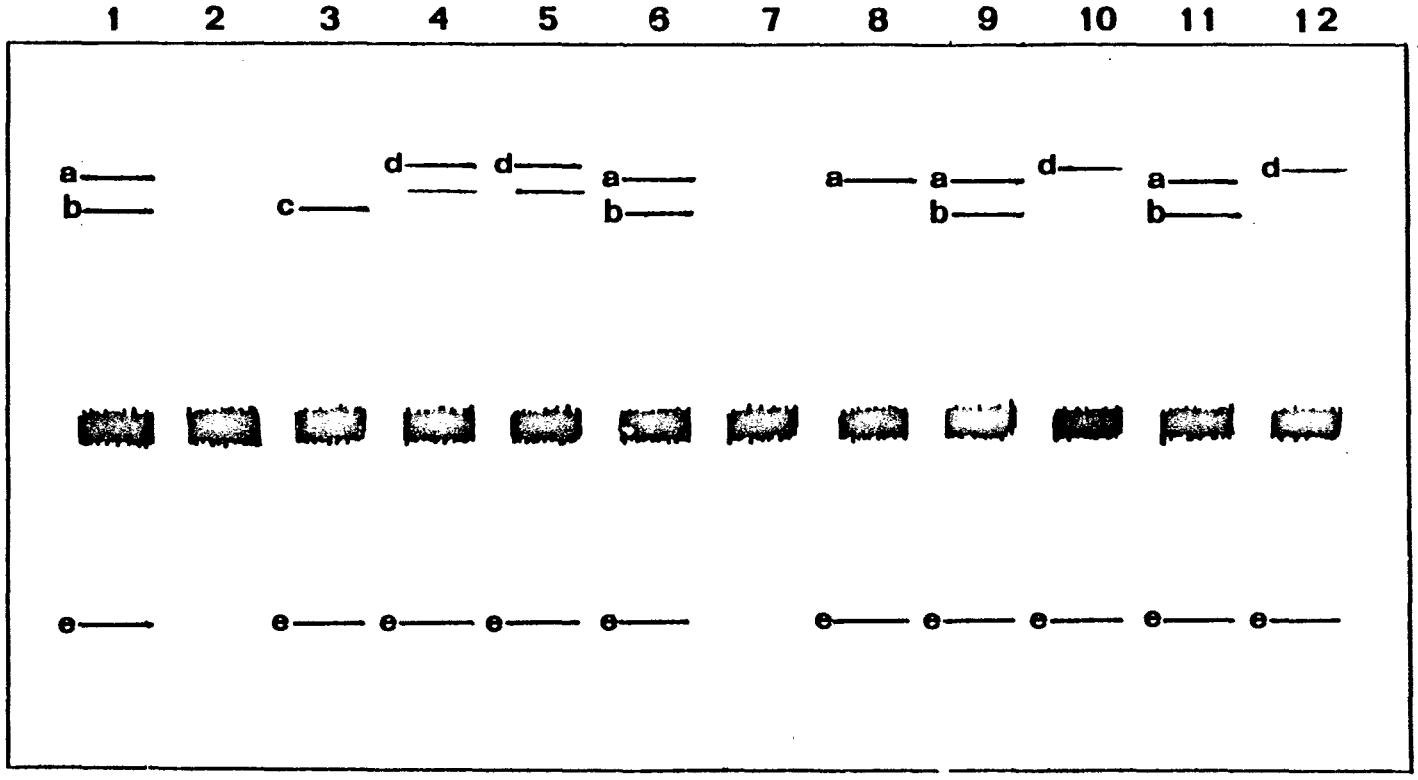


FIG. 12. Schematic representation of agarose gel electrophoresis of plasmid DNA from B. anthracis pXO1 donors and B. anthracis recipients and transcipients. Plasmid bands: a, pXO1; b, pXO12; c, probable recombinant plasmid of pXO1 and pXO12; d, probable cointegrate plasmid of pXO1 and pXO12, e, pBC16. The large diffuse band in all lanes is chromosomal DNA. Lanes: 1, UM17 tr57B-6, pXO1 donor; 2, UM44-1 C9, recipient; 3, UM44-1 C9 tr156G-2, transcipient; 4, UM44-1 C9 tr156G-5, transcipient; 5, UM44-1 C9 tr156G-6, transcipient; 6, UM2 tr244-1, pXO1 donor; 7, UM23 C1-2, recipient; 8, UM23 C1-2 tr201G-1, transcipient; 9, UM23 C1-2 tr201G-2, transcipient; 10, UM23 C1-2 tr220G-2, transcipient; 11, UM23 C1-2 tr220G-3, transcipient; 12, UM23 C1-2 tr220G-4, transcipient.

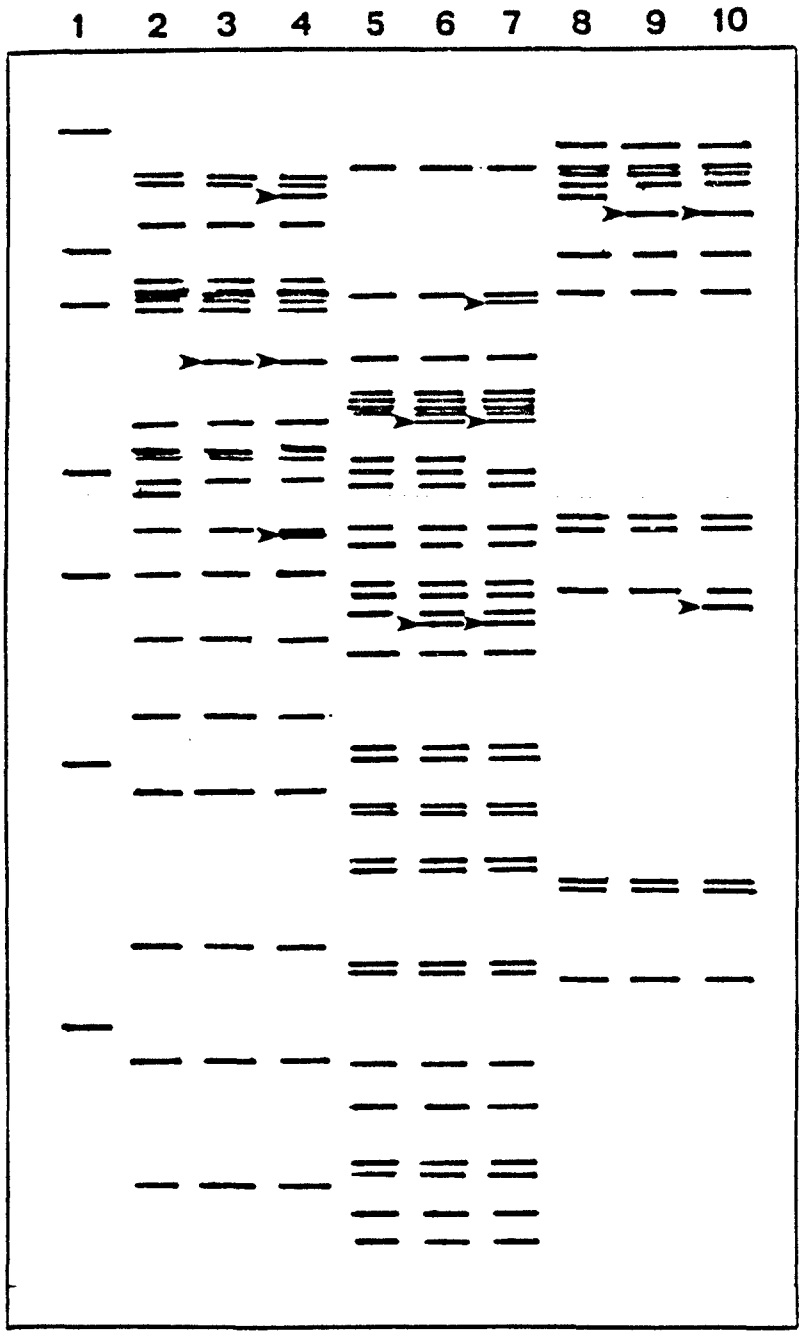


FIG. 13. Agarose gel electrophoresis of pXO1, pXO1.1, and pXO1.3 digested with PstI, EcoRI, and KpnI. Lanes 1-4 digested with PstI; lanes 5-7 digested with EcoRI; lanes 8-10 digested with KpnI. Lane 1, pXO12; lanes 2, 5, 8, pXO1; lanes 3, 6, 9, pXO1.1; lanes 4, 7, 10, pXO1.3. The arrows indicate altered fragments which showed homology to pXO12.

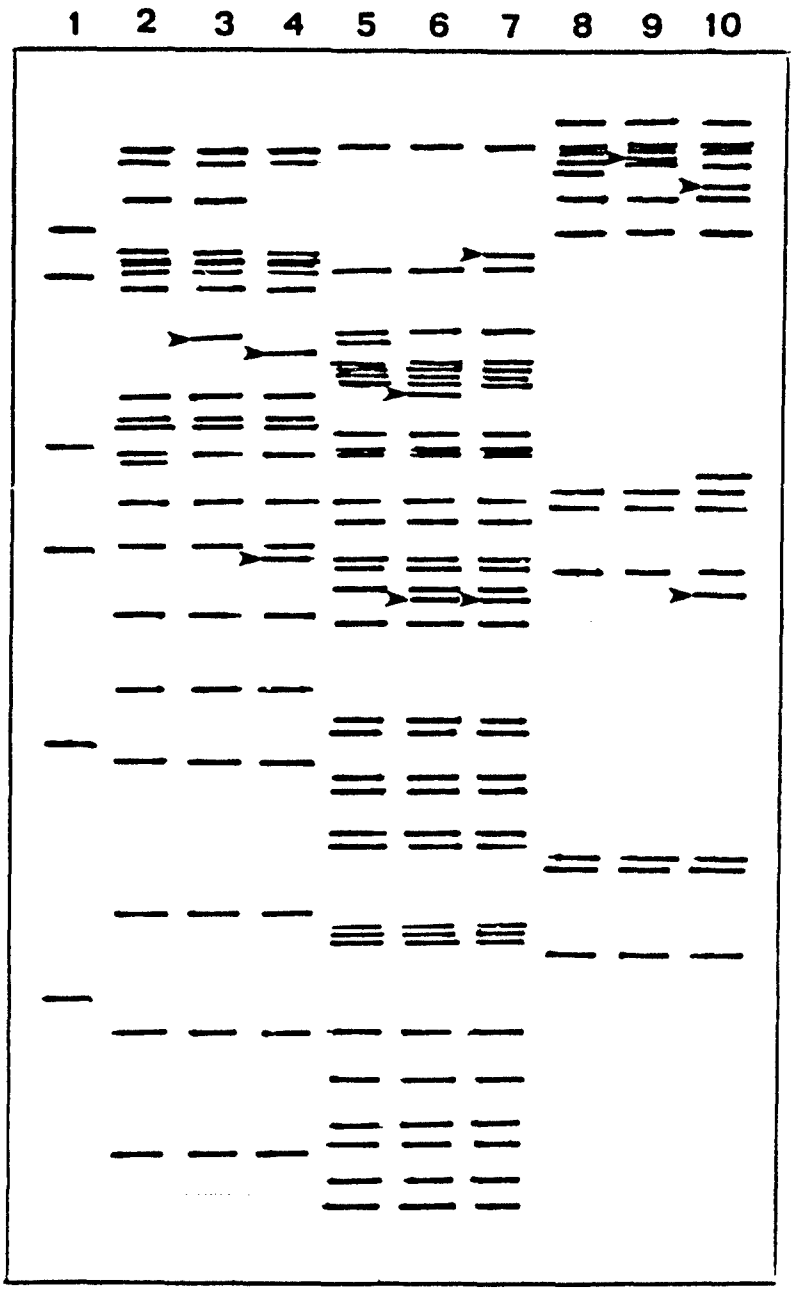


FIG. 14. Agarose gel electrophoresis of pXO1, pXO1.1, and pXO1.2 digested with PstI, EcoRI, and KpnI. Lanes 1-4 digested with PstI; lanes 5-7 digested with EcoRI; lanes 8-10 digested with KpnI. Lane 1, pXO12; lanes 2, 5, 8, pXO1; lanes 3, 6, 9, pXO1.1; lanes 4, 7, 10, pXO1.2. The arrows indicate altered fragments which showed homology to pXO12.

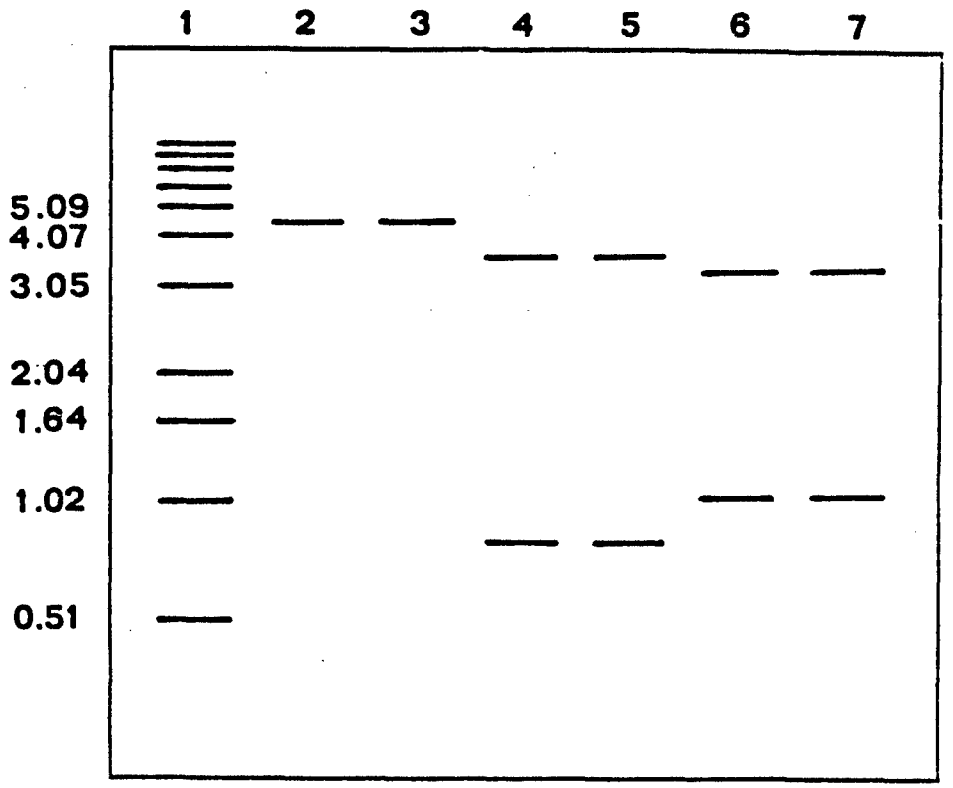


FIG. 15. Agarose gel of 4.2 kb KpnI fragment from pXO12 and pHT44.
Lane 1. BRL kb ladder
Lanes 2, 4, and 6. 4.2 kb fragment from pHT44.
Lanes 3, 5, and 7. 4.2 kb fragment from pXO12.
Lanes 4 and 5 were digested with PstI. Lanes 6 and 7 were digested with HindIII.
The numbers on the left represent fragment sizes in kilobases.

PUBLICATIONS

The following abstract and papers were published or accepted for publication during this reporting period:

1. Koehler, T. M., and C. B. Thorne. Bacillus subtilis (natto) plasmid pLS20 mediates interspecies plasmid transfer. Abstr. Annu. Meet. Am. Soc. Microbiol. H-105, p. 157. 1987.

2. Reddy, A., L. Battisti, and C. B. Thorne. Identification of self-transmissible plasmids in four Bacillus thuringiensis subspecies. J. Bacteriol. (In press).

3. Koehler, T. M., and C. B. Thorne. Bacillus subtilis (natto) plasmid pLS20 mediates interspecies plasmid transfer. J. Bacteriol. (In press).

The following Ph. D. dissertation was written on research carried out under this contract:

Reddy, Amala. Characterization of four self-transmissible Bacillus thuringiensis plasmids. Ph. D. Dissertation. University of Massachusetts, Amherst. February 1987.

LITERATURE CITED

1. Aronson, A. I., and W. Beckman. 1987. Transfer of chromosomal genes and plasmids in Bacillus thuringiensis. Appl. Environ. Microbiol. 53:1525-1530.
2. Aronson, A. I., W. Beckman, and P. Dunn. 1986. Bacillus thuringiensis and related insect pathogens. Microbiol. Rev. 50:1-24.
3. Aronson, A. I., W. Beckman, and S. A. Minnich. 1984. Regulation of Bacillus thuringiensis protoxin production. in A. T. Ganesan and J. A. Hoch (ed.), Genetics and biotechnology of Bacilli. Academic Press, New York.
4. Battisti, L., B. D. Green, and C. B. Thorne. 1985. Mating system for transfer of plasmids among Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis. J. Bacteriol. 162:543-550.
5. Bernhard, K., H. Schrempf, and W. Goebel. 1978. Bacteriocin and antibiotic resistance plasmids in Bacillus cereus and Bacillus subtilis. J. Bacteriol. 133:897-903.
6. Carlton, B. C., and J. M. Gonzalez, Jr. 1984. The genetics and molecular biology of Bacillus thuringiensis. In D. A. Dubnau (ed.), The molecular biology of the bacilli, Vol. 2. Academic Press, New York.
7. Chang, S. and S. N. Cohen. 1979. High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA. Molec. Gen. Genet. 168:111-115.
8. Clark, A. J., and G. J. Warren. 1979. Conjugal transmission of plasmids. Ann. Rev. Genet. 13:99-125.
9. Faust, R. M., C. F. Reichelderfer, and C. B. Thorne. 1981. Possible uses of recombinant DNA for genetic manipulations of entomopathogenic bacteria. In N. J. Panopoulos (ed), Genetic engineering in the plant sciences. Praeger, New York.
10. Gryczan, T. J., Contente, S., and Dubnau, D. 1978. Characterization of Staphylococcus aureus plasmids introduced by transformation into Bacillus subtilis. J. Bacteriol. 134:318-329.
11. Gryczan, T. J., A. G. Shivakumar, D. Dubnau. 1980. Characterization of chimeric plasmid cloning vehicles in Bacillus subtilis. J. Bacteriol. 141:246-253.
12. Hara, T., A. Aumayr, Y. Fujio, and S. Ueda. 1982. Elimination of plasmid-linked polyglutamate production by Bacillus subtilis (natto) with

acridine orange. Appl. Environ. Microbiol. 44:1456-1458.

13. Hara, T., A. Aumayr, and S. Ueda. 1981. Characterization of plasmid deoxyribonucleic acid in Bacillus natto: Evidense for plasmid-linked PGA production. J. Gen. Appl. Microbiol. 27:299-305.
14. Hara, T., J. -R. Zhang, and S. Ueda. 1983. Identification of plasmids linked with polyglutamate production in Bacillus subtilis (natto). J. Gen. Appl. Microbiol. 29:345-354.
15. Heierson, A., R. Landen, A. Lovgren, G. Dalhammar, and H. G. Boman. 1987. Transformation of vegetative cells of Bacillus thuringiensis by plasmid DNA. J. Bacteriol. 169:1147-1152.
16. Kado, C., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.
17. Kronstad, J. W., and H. R. Whiteley. 1984. Inverted repeat sequences flank a Bacillus thuringiensis crystal protein gene. J. Bacteriol. 160:95-102.
18. Lereclus, D., M. -M. Lecadet, J. Ribier, and R. Dedonder. 1982. Molecular relationships among plasmids of Bacillus thuringiensis: conserved sequences through 11 crystalliferous strains. Mol. Gen. Genet. 186:391-398.
19. Lereclus, D., J. Mahillon, G. Menou and M. -M. Lecadet. 1986. Identification of Tn4430, a transposon of Bacillus thuringiensis functional in Escherichia coli. Mol. Gen. Genet. 204:52-57.
20. Lereclus, D., G. Menou, and M. -M. Lecadet. 1983. Isolation of a DNA sequence related to several plasmids from Bacillus thuringiensis after a mating involving the Streptococcus faecalis plasmid pAMβ1. Mol. Gen. Genet. 191:307-313.
21. Lereclus, D., J. Ribier, A. Klier, G. Menou, and M. -M. Lecadet. 1984. A transposon-like structure related to the endotoxin gene of Bacillus thuringiensis. EMBO 3:2561-2567.
22. Mahillon, J., Seurinck, L. Van Rompuy, J. Delcour, and M. Zabeau. 1985. Nucleotide sequence and structural organization of an insertion sequence element (IS231) from Bacillus thuringiensis strain berliner 1715. EMBO 4:3895-3899.
23. Minnich, S. A., and A. Aronson. 1984. Regulation of protoxin synthesis in Bacillus thuringiensis. J. Bacteriol. 158:447-454.
24. Perkins, J. B. and P. J. Youngman. 1984. A physical and functional analysis of Tn917, a Streptococcus transposon in the Tn3 family that functions in Bacillus. Plasmid 12:119-138.

25. Polack, J., and R. P. Novick. 1982. Closely related plasmids from Staphylococcus aureus and soil bacilli. Plasmid 7:152-162.

26. Reddy, A., L. Battisti, and C. B. Thorne. 1986. Identification of self-transmissible plasmids in four Bacillus thuringiensis subspecies. J. Bacteriol. In press.

27. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1980. Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.

28. Ruhfel, R. E., N. J. Robillard, and C. B. Thorne. 1984. Interspecies transduction of plasmids among Bacillus anthracis, B. cereus, and B. thuringiensis. J. Bacteriol. 157:708-711.

29. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

30. Tanaka, T. and T. Koshikawa. 1977. Isolation and characterization of four types of plasmids from B. subtilis (natto). J. Bacteriol. 131:699-701.

31. Thorne, C. B. 1968. Transducing bacteriophage for Bacillus cereus. J. Virol. 2:657-662.

32. Thorne, C. B. 1968. Transduction in Bacillus cereus and Bacillus anthracis. Bacteriol. Rev. 32:358-361.

33. Thorne, C. B. 1978. Transduction in Bacillus thuringiensis. Appl. Environ. Microbiol. 35:1109-1115.

34. Thorne, C. B. Annual Progress Report. Contract DAMD17-80-C-0099. December 1984.

35. Thorne, C. B. 1985. Genetics of Bacillus anthracis, p. 56-62. In L. Leive (ed.), Microbiology - 1985. American Society for Microbiology, Washington, D.C.

36. Thorne, C. B. Annual Progress Report and Final Report. Contract DAMD17-80-C-0099. July 1985.

37. Thorne, C. B., and F. C. Belton. 1957. An agar diffusion method for titrating Bacillus anthracis immunizing antigen and its application to a study of antigen production. J. Gen. Microbiol. 17:505-516.

38. Vandeyar, M. A. and S. A. Zahler. 1986. Chromosomal insertions of Tn917 in Bacillus subtilis. J. Bacteriol. 167:530-534.

39. Weisblum, B., M. Y. Graham, T. Gryczan, and D. Dubnau. 1979. Plasmid copy number control: Isolation and characterization of high-copy-number mutants of plasmid pE194. J. Bacteriol. 137:635-643.

40. Yelton, D. B., and C. B. Thorne. 1970. Transduction in Bacillus cereus by each of two bacteriophages. J. Bacteriol. 102:573-579.

41. Youngman, P. J., J. B. Perkins, and R. Losick. 1983. Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in Bacillus subtilis or expression of the transposon-borne erm gene. Plasmid 12:1-9.

42. Youngman, P. J., J. B. Perkins, and K. Sandman. 1984. New genetic methods, molecular cloning strategies and gene fusion techniques for Bacillus subtilis which take advantage of Tn917 insertional mutagenesis. In A.T. Ganeson and J.A. Hoch (eds.), Genetics and Biotechnology of Bacilli. Academic tress.

DISTRIBUTION LIST

5 copies	Commander US Army Medical Research Institute of Infectious Diseases ATTN: SGRD-UIZ-E Fort Detrick Frederick, MD 21701-5011
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RM1-S Fort Detrick Frederick, MD 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100